Electrophoretic and other studies of the surface of some fungal spores.

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ELECTROPHORETIC AND OTHER STUDIES OF THE SURFACE OF SOME FUNGAL SPORES

submitted by David John Fisher

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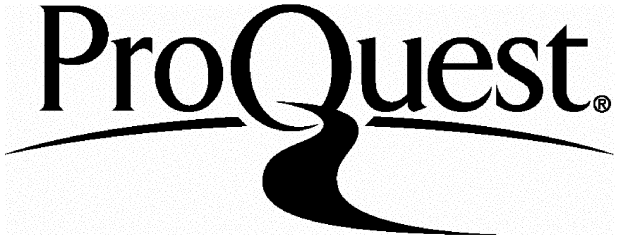

SUMMARY

The microelectrophoretic technique has been used in conjunction with chemical and enzymic treatments to investigate the surface composition of fungal spores. The surface reactions of some cationic fungicides are also considered.

Simple carboxyl surfaces, amino-carboxyl surfaces, complex phosphate-containing surfaces, and a surface free of ionogenic material have been identified. The spores of closely related species frequently have widely different surface compositions. Stabilised fungal protoplasts have an amino-carboxyl surface probably derived from protein or lipoprotein.

Electrophoretic measurements carried out in the presence of sodium dodecyl sulphate showed lipid to be absent from most of the spores examined. Thin-layer and gas-liquid chromatography showed that lipid on the spore surface differed from lipid extracted from the spore wall. Surface and wall fatty acids had similar major components but the corresponding hydrocarbons differed both quantitatively and qualitatively.

A superficial polyphosphate layer on the surface of Penicillium expansum conidia has been demonstrated by microelectrophoresis and confirmed by chemical analysis. The layer is absent from freshly formed spores and its composition is dependent on the phosphate content of the medium. A rodlet layer beneath the polyphosphate is free of cutin and, unlike similar structures on bacterial spores, does not consist of a unique protein. In contrast to the polyphosphate layer the rodlets form an integral part of the wall.

A toxic concentration of the surface-active fungicide dodine completely neutralises the charge on protoplasts but has no detectable effect on cell walls. There is no evidence that the toxic reaction is at the spore surface; changes at the protoplast membrane are more likely to be the cause of death.

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INTRODUCTION

PART I

THE FUNGAL SPORE SURFACE

A. The Fungal Spore

Production of viable spores is the principal method of perpetuation of fungi. Spores may be divided into two groups: those such as zygospores which remain at their place of origin and others like conidospores which initiate new mycelium at a distance. The surface structure of the second group, variously called "Summer spores", "Diaspores", or "Xenospores" (Gregory, 1966), is of paramount importance in relation to dispersal and survival.

Fungal spores vary in size, shape, and colour; in the number and arrangement of their component cells, and in the manner in which they are borne. Spore surfaces differ enormously in complexity, ranging from the naked zoospores of water moulds devoid of a true cell wall, through the smooth cellulose surface of encysted zoospores to the deeply warted structures typified by rust uredospores. Spores may be divided into "dry" and "slime spore" types. This surface quality governs the mode of dispersion by air, water, soil, or insect vectors. The importance of the study of the surface layers of cell walls rather than a general study of the whole wall is thus evident.

B. Methods of examining spore surfaces

The larger ornamentations of spore cell walls may be distinguished by light microscopy. Because of the high density of spores to electrons direct examination in the electron microscope shows only an outline. The carbon replica technique outlined by Bradley and Williams (1957) may however be used

to study the spore surface (Bigelow and Rowley, 1968; Marchant, 1966).

Surface structures may be seen in three dimensions with the scanning electron microscope. A minimum of preparation is required but resolution is poor compared with that obtained with the transmission electron microscope. Several workers (Hawker, Thomas and Beckett, 1970; Campbell, 1969; Wyllie and Brown, 1970; Brown and Wyllie, 1970) have used the scanning electron microscope to study fungal surfaces and Hayes and Pease (1968) have reviewed the uses of the instrument in biology. Much greater detail may be observed using the freeze etching technique of Moor (1966). The object is frozen alive, and a replica, obtained by a procedure of fracturing, etching and coating, is examined in an electron microscope. The method is reviewed by Koehler (1968). The technique has revealed in detail the spore surface of Penicillium species (Hess, Sassen and Remsem, 1968) and Aspergillus species (Hess and Stocks, 1969).

While electron microscopic techniques give an indication of the surface topography they provide little evidence of the chemical composition. Studies of the overall chemical composition of fungal cell walls give a general picture of their qualitative and quantitative components but give no information as to their arrangement. Many of the components of fungal walls contain ionogenic groups such as carboxyl groups from proteins and lipids, amino groups from proteins and amino-sugars, and phosphate groups from phospholipids. Using the microelectrophoretic technique it is possible to determine the charged groups present on the surface and the class of substance from which they are derived. Changes in surface charge brought about by reaction with fungicides can also be investigated.

PART II

MICROELECTROPHORESIS

A. Theory of microelectrophoresis

Biological particles in aqueous suspensions acquire a charge due to the ionisation of their surface groups and adsorption of ions. The electrokinetic behaviour depends on the potential known as the zeta potential, at the plane of shear between the charged surface and the electrolyte solution. The theory of Stern (1924), which embodies several earlier concepts, postulates a double layer distribution of ions at the interface. A layer approximately a single ion in thickness is almost in contact with the surface and is surrounded by a more diffuse ion cloud. The value of the zeta potential depends on the position of the shearing plane with respect to the two layers.

Movement caused by an applied potential gradient is expressed as the electrophoretic mobility. Smoluchowski (1914) related this to the zeta potential by the equation:-

$$\bar{V} = \frac{D\zeta}{4\pi\eta} \quad \ldots\ldots \quad \text{(Equation 1)}$$

where $\bar{V}$ = the electrophoretic mobility of the particle.

D = the dielectric constant of the medium .

ζ = the potential at the plane of shear.

η = the viscosity of the medium.

This relationship applies where the ratio of surface curvature to double layer thickness is large. Its application to particles of the dimensions of fungal spores has been confirmed by Brinton and Lauffer (1959). Most workers have preferred to report their results as electrophoretic mobilities.

B. Measurement of electrophoretic mobilities

The migration velocity of particles under an applied field

may be measured by studying the movement of the boundary formed between a colloidal suspension and the dispersion medium, or by observing individual cells under the microscope. The moving boundary technique has found particular application in the study of proteins. The microscope method, first used by Ellis (1911), is known as cell electrophoresis or micro-electrophoresis. A comparison of the two methods and of the mathematical factors involved has been made by Shaw (1969).

An important advantage of microelectrophoresis is that the size, shape and orientation of the particles can be observed. Thus in a mixed population measurements can be carried out on definitely identified particles. Further benefits lie in simplicity of the apparatus and speed of operation. The method is however less useful for the study of concentrated dispersions. The zeta potential is sensitive to changes in ionic strength of the suspension medium, which must therefore be rigidly defined (Barry and James, 1952). The method has been used for characterising the surface of bacteria (James, 1957), blood cells (Seaman and Uhlenbruck, 1963), viruses (Douglas, Rondle and Williams, 1966), plant cells (Lukiewicz and Korohoda, 1965b), animal cells (Ambrose, James and Lowick, 1956), protoplasts (James, Hill and Maxted, 1965) and sub-cellular particles (Vassar, Seaman, Dunn and Kanke, 1967). Microelectrophoretic behaviour may be influenced by diffusion of ions through the cell membrane (James, Loveday and Plummer, 1964), or by the presence of mucilages, capsules or fimbriae (James and List, 1966). The surface defined by microelectrophoresis does not therefore necessarily coincide with that observed by light or electron microscopy.

A microelectrophoresis apparatus consists essentially of a cell into which a microscope can be focussed, electrodes, and an arrangement for filling and emptying the cell. Provision must be made for efficient stabilisation of cell temperature since particle velocity is affected by viscosity of the medium (Powney and Wood, 1940) and convection currents. The numerous cells which have been described (see Brinton and Lauffer, 1959) fall into two main categories - rectangular and cylindrical. A cell of rectangular cross section is preferable for particles tending to sediment, such as fungal spores. The relative merits of the two types have been discussed by Shaw (1969). Reviews of microelectrophoresis carried out in cylindrical cells and discussions of the mathematical implications have been made by Bangham, Flemans, Heard and Seaman (1958) and Seaman (1965).

Reversible electrodes are desirable with equipment containing a rectangular cell in order to avoid gas evolution and polarisation. Douglas (1947) described an apparatus incorporating these features which was subsequently used to study fungal spores (Douglas, Collins and Parkinson, 1959). Copper/copper sulphate electrodes were separated from the liquid in the cell by filter paper plugs. Plugs of plaster of Paris or agar have also been used (Hannan, 1961), but the most effective method of separation is the use of sintered glass disks (Loveday and James, 1957). Gittens and James (1960) constructed an apparatus using this system with silver/silver chloride electrodes and a rectangular cell mounted horizontally. Under rigorously standardised conditions

the velocity of bacterial cells was reproducible to ±3% over a period of weeks. A modification of this apparatus has been used in the work to be described.

The walls of an electrophoresis cell assume a charge relative to the suspension medium, causing electro-osmotic streaming in the closed system (van Gils and Kruyt, 1937). Liquid flow proceeds along the walls and back through the centre of the cell (Figure 1). There is no known method of eliminating this. The true electrophoretic speed of the particles is observed at only two levels, the so-called 'stationary levels', where the electro-osmotic flow and return flow of liquids exactly cancel. Electrophoretic measurements are almost invariably made at one or other of these stationary levels. Calculation of the position of the stationary levels is complex but it is generally agreed (see Douglas, 1947) that for rectangular cells they lie at .21 and .79 of the total depth. These positions have been confirmed experimentally using particles of known electrophoretic mobility (Fuhrmann and Ruhenstroth-Bauer, 1965; Shaw, 1969). Figure 2 shows the parabolic profile of the mobility-depth curve used by Hartman, Bateman and Lauffer (1952) to establish cell symmetry.

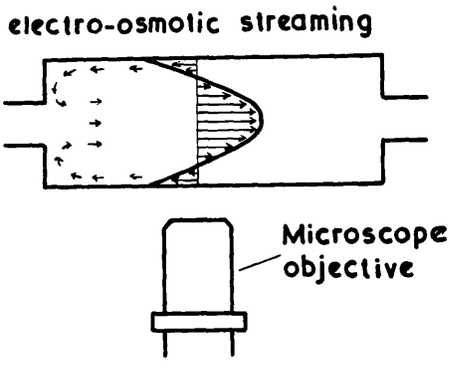


Figure 1. Electro-osmotic streaming in a closed rectangular electrophoresis cell.

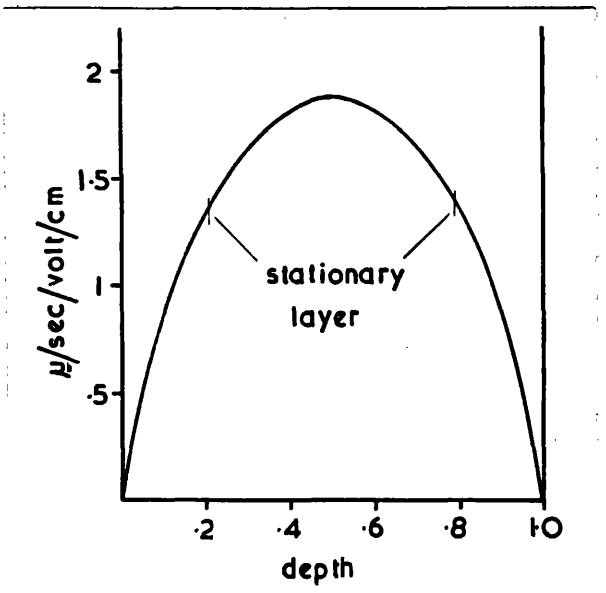


Figure 2. Mobility of human erythrocytes as a function of fractional cell depth (From Lukiewicz and Korohoda, 1965a).

C. Identification of surface groups

The cell surface may be altered by changing the composition of the suspension medium. This provides a method for identifying surface groups. Surface phosphate groups may be identified by the reduction in mobilities produced in the presence of uranyl ions (McQuillen, 1950b) or calcium ions (Forrester, Dumonde and Ambrose, 1965). Surface lipids react with the hydrophobic moiety of anionic surfactants leaving the polar groups oriented towards the aqueous phase. The negative charge at the cell surface is thus increased (Dyar and Ordal, 1946). The surfactant sodium dodecyl sulphate has been used in investigations on bacteria (Hill, James and Maxted, 1963b), protoplasts (James et al., 1965), and actinomycete spores (Douglas, Ruddick and Williams, 1970). The reaction of 1,2-cis-hydroxyl groups with borates was utilised by Douglas et al. (1970) in surface carbohydrate investigations. A "Charge reversal spectrum" showing the order of effectiveness

of a series of cations in reversing charges has also been employed to characterise the outer surface layers of bacteria (Davies, Haydon and Rideal, 1956; Douglas and Parker, 1957), and blood cells (Bangham, Pethica and Seaman, 1958). However different surface groups may give similar spectra. The strongly acid surface of erythrocytes, initially considered to originate from phospholipid phosphoric acid groups by a study of the charge reversal spectrum, was subsequently shown to be due to sialic acids (Seaman and Cook, 1965).

Electrophoretic mobility varies with pH. The form of the pH/mobility curve, coupled with the isopotential value, may be used to distinguish between surfaces. Identification of surface ionogenic groups may also be made by a comparison of pH/mobility curves with those obtained from model particles of known surface composition (Douglas and Shaw, 1957; Douglas et al., 1959; Plummer and James, 1961). Surface groups may also be identified by comparing pH/mobility plots of untreated cells with those obtained from material changed by specific chemical and enzymic treatments. Cohen (1945) first employed this method when he modified primary and secondary amino groups on the surface of Bacillus proteus cells with benzenesulphonyl chloride. Similar use has been made of p-toluenesulphonyl chloride by Douglas (1959) and of 1-fluoro-2,4-dinitrobenzene by Gittens and James (1963). Surface carboxyl groups may be methylated with diazomethane (Maccaro and James, 1959) or methanolic hydrogen chloride (Gittens and James, 1963). Carboxyl groups also react with ethyleneimine to form β-amino esters (Gittens and James, 1963). Other reagents employed with varying degrees of success include

nitrous acid and ninhydrin for amino groups (Douglas, 1959), and propylene and ethylene oxides for esterification of carboxyl groups (Gittens and James, 1963). A similar technique may be used to demonstrate the presence of enzyme substrates on surfaces. Enzymes such as lysozyme, hyaluronidase and lipase were employed by Douglas and Parker (1958a) while Hill, James and Maxted (1963a) have used proteases and alkaline phosphatase. Chemical and enzymic modification of bacterial surfaces has been reviewed by James (1965a).

D. Microelectrophoresis of Fungi

Jansen and Mendlik (1951) found little difference in the charge reversal spectrum of flocculating and powdery strains of Saccharomyces cerevisiae. The results correspond to a carboxyl surface. Wiles (1951) measured the mobilities of a wide range of brewers yeasts and concluded that insoluble material may be deposited during fermentation so modifying the surface charge. Eddy and Rudin (1958) found that the mobility at pH 4.0 of various strains of S.cerevisiae and Saccharomyces carlsbergensis could be correlated with the phosphorus content of the cell walls. Briley, Illingworth, Rose and Fisher (1970) used the microelectrophoretic technique to investigate the surface of S.cerevisiae ascospores and presented evidence for a surface protein layer.

Little attention has been given to the electrophoretic properties of fungi apart from yeasts. Asexual spores of four fungal species were examined by Douglas et al. (1959) who compared the surfaces of dry spores of two Penicillium species with the slime spores from Mucor ramannianus and Fusarium lini. All spore

velocities were shown to have unimodal distributions and the possible use of electrophoretic behaviour as a taxonomic aid was suggested. Hannan (1961) studied the surface components of Aspergillus niger spores and concluded that lipids and proteins were absent and that the surface was of a polysaccharide nature. With the exception of work by the present author no other papers are known to exist concerning the microelectrophoresis of fungi.

E. Microelectrophoresis of Protoplasts

Protoplasts may be defined as cell contents enclosed by the thin protoplasmic membrane only (Brenner, Dark, Gerhardt, Jeynes, Kandler, Kellenberger, Klieneberger-Nobel, McQuillen, Rubio-Huertos, Salton, Strange, Tomcsik and Weibull, 1958). Spheroplasts are similar though fragments of cell wall may still be present (Villanueva, 1966). Both are osmotically fragile. Douglas and Parker (1958b) used sucrose-containing buffers to stabilise bacterial protoplasts during velocity measurements. Electrophoretic mobilities were corrected to correspond with a medium of the same viscosity as water. This method was also used by Few, Gilby and Seaman (1960) who compared sucrose-stabilised bacterial protoplasts with the membranes derived from them. The similarity in electrokinetic properties led these workers to the conclusion that it was simpler to study ruptured membranes than stabilised protoplasts. Gebicki and James (1962) have pointed out that high sucrose concentrations cause changes in viscosity and dielectric constant which render interpretation of electrophoretic measurements difficult and unreliable. The possibility of surface adsorption cannot be ignored. Sucrose concentrations below 0.3M had however little

effect on the electrophoretic mobilities of bacterial spheroplasts. James et al.,(1965) have also studied the surface of "protoplast" bodies, osmotically strengthened by heat coagulation before removal of the cell wall. All electrokinetic studies of protoplasts and spheroplasts indicate protein or lipoprotein surfaces.

PART III

THE CHEMICAL COMPOSITION OF FUNGAL CELL WALLS

A. Polysaccharides

Studies of the chemistry of fungal cell walls date from the work of Bracconot (1811) who isolated "fungine" an impure form of chitin. Investigations have been mainly concerned with hyphal walls which are principally composed of polysaccharide. Much of the early work was concerned with distinguishing between cellulose and chitin. Basidiomycetes and most Ascomycetes were found to have chitinous walls. Wall components varied in the Phycomcetes; Zygomycetes and Chytridiomycetes contained chitin and Oomycetes contained cellulose (Cochrane, 1958). More recently it has been demonstrated that Hypochytridiomycetes constitute an intermediate group of Phycomycetes in which both cellulose and chitin occur (Fuller and Barshad, 1960; Fuller, 1960). The only higher fungus in which both cellulose and chitin have been definitely identified is Ceratocystis ulmi (Rosinski and Campana, 1964). The fibrous structure of fungal cell walls has been firmly established by X-ray diffraction (van Iterson, Meyer and Lotmar, 1936) and electron microscopy (e.g. Manocha and Colvin, 1967; Scurfield and Da Costa, 1969; Dodge and Lawes, 1969). Chitin or cellulose are the main components of the microfibrils which are embedded in a matrix of other wall constituents. Following the introduction of mechanical methods of preparation and of improved analytical techniques it became clear that other polysaccharides occur in the cell wall. Principal polysaccharide components are chitin (β-1,4 linked N-acetyl-D-glucosamine units), chitosan (a completely or partially deacetylated form of chitin), cellulose (β-1,4 linked D-glucose units), various glucans (mostly β-1,3 and β-1,6 linked) and incompletely characterised mannans

and glycogen-like polymers (Bartnicki-Garcia and Reyes, 1968a; Crook and Johnston, 1962; Hamilton and Knight, 1962; Parker, Preston and Fogg, 1963). Tanaka, Ogasawara, Nakajima and Tamari (1970) suggested that β-1,3 glucans are important in maintaining cell wall morphology but Hunsley and Burnett (1970) concluded that the mechanical integrity of cell walls does not reside in any one major constituent. Bartnicki-Garcia (1968) has proposed a taxonomic system based on the dual combinations of these polysaccharides in walls of vegatative cells.

The presence of uronic acids in cell walls has recently been demonstrated. Gancedo, Gancedo and Asensio (1966) detected oligomers of D-glucuronic acid in cell wall hydrolysates of various higher fungi, whilst a heteropolysaccharide containing D-glucuronic acid, D-galactose, D-glucose and D-mannose has been isolated from the cell wall of the Deuteromycete Aureobasidium pullulans (Brown and Lindberg, 1967). Bartnicki-Garcia and Reyes (1968b) found 25% of the sporangiophore wall of Mucor rouxii to consist of D-glucuronic acid polymers. Polysaccharides may also be present on the surface of the cell wall as capsules or mucilages. Probable structures have been reviewed by Nordin and Kirkwood (1965). Marchant (1966) considered the capsule around Fusarium culmorum conidia to be composed of xylan, on the basis of cytochemical tests. Extracellular polysaccharides produced by Claviceps species contain branched glucans with mainly β-1,3 linkages (Buck, Chen, Dickerson and Chain, 1968) and similar material is present on the hyphae of the marine Ascomycete Leptosphaeria albopunctata (Szaniszlo, Wirsen and Mitchell, 1968).

Few investigations have dealt specifically with spore wall composition. Chitin is the principal polysaccharide in spore walls of Neurospora sitophila (Owens, Novotny and Michels, 1958). Rizza and Kornfeld (1969) found the carbohydrate composition of hydrolysates of conidial and hyphal walls of Penicillium chrysogenum to be similar. Bartnicki-Garcia and Reyes (1964) however showed glucans to be the main constituents of Mucor rouxii sporangiospore walls, contrasting with hyphal walls where chitosan predominates.

B. Lipids

The lipid content of fungal cell walls varies from 1-2% in hyphal walls of Penicillium notatum (Applegarth, 1967) to 25% in sporangiophore walls of Phycomyces blakesleeanus (Kreger, 1954). The distinction between readily extractable and complex bound fractions has been made by Nickerson (1963) who considers that lipids may play a part in the architecture of the yeast cell wall. Bartnicki-Garcia and Reyes (1968a) suggest that macromolecular complexes of polysaccharides, proteins and lipids, obscure precise molecular boundaries. This may explain the marked differences in the lipid content of Aspergillus niger walls prepared by different mechanical methods (Johnston, 1965).

Qualitative studies of the lipids of whole cells are numerous but few attempts have been made to isolate and analyse wall fractions. Dyke (1964) showed that fatty acids were present in the cell wall of the yeast Nadsonia elongata in proportions different from those in whole cells. Palmitic, stearic and oleic acids were major components of the wall. Analysis of the cell wall of Pithomyces chartarum by

Russell, Sturgeon and Ward (1964) showed palmitic, oleic, linoleic, stearic and palmitoleic acids to be present. Bertaud, Morice, Russell and Taylor (1963) found benzene washings of P.chartarum spores differed in composition from the total spore lipids and concluded that extracted material was derived from the surface. Oleic, linoleic and palmitic acids were major surface components. These acids were also present on the spore surface of the smut Ustilago maydis (Laseter, Weete and Weber, 1968b). Oró, Laseter and Weber (1966) found alkanes of the odd carbon series in the range C_{25} - C_{31} on the surface of U.maydis, Ustilago nuda and Sphacelotheca reiliana chlamydospores. A similar alkane distribution has been reported in a surface extract from spores of three Tilletia species (Laseter, Hess, Weete, Stocks and Weber, 1968a).

C. Proteins

Several early investigators (Thomas, 1928; Richards, 1954) concluded that protein was present in fungal cell walls, mainly on the basis of cytochemical tests. More recently, using mechanically isolated cells, Aronson and Machlis (1959) found as much as 10% protein in purified hyphal walls of Allomyces macrogynus. Drastic extraction methods failed to remove protein from hyphal walls of Phytophthora species (Bartnicki-Garcia, 1966), Mucor rouxii (Bartnicki-Garcia and Nickerson, 1962a), Aspergillus species (Ruiz-Herrera, 1967) and Penicillium notatum (Applegarth, 1967). Protein thus appears to be an integral part of the wall structure. Protein complexes with pigments and glucosamine are present in M.rouxii spore walls (Bartnicki-Garcia and Reyes, 1964) and

with glucans in Neurospora crassa hyphal walls (Manocha and Colvin, 1967). Fungal wall proteins apparently contain a full complement of amino acids (Crook and Johnston, 1962; Shah and Knight, 1968; Aronson and Fuller, 1969).

D. Inorganic constituents

Considerable amounts of inorganic compounds occur in fungal walls. Aronson and Machlis (1959) found 10% ash in the hyphal walls of Allomyces macrogynus. Hyphal walls of Penicillium digitatum contained 29.5% ash contrasting with less than 0.5% in Penicillium italicum walls (Grisaro, Sharon and Barkai-Golan, 1968). The ash content of Mucor rouxii walls was found to be 18.2% for the filamentous form and 15.6% for the yeast-like form. Phosphorus, magnesium and calcium are the major constituents of the ash although smaller quantities of silicon and iron, and traces of copper, chromium, aluminium, cobalt, barium, manganese and sodium are also present (Bartnicki-Garcia and Nickerson, 1962a). Inorganic components in fungal walls may be a reflection of the binding capacity of the surface. Sussman, von Böventer-Heidenhain and Lowry (1957) studied the uptake of a variety of cations by Neurospora tetrasperma ascospores; up to 11% of the total uptake may be bound to the cell wall. The binding of inorganic polyphosphate ions to polygalactosamine and proteins in hyphal walls of Neurospora crassa has been demonstrated by Harold (1962a).

E. Other components

Nucleic acids have been detected in acid hydrolysates of cell walls of the yeast-like and filamentous forms of Mucor rouxii by ultraviolet spectroscopy. Adenine, guanine,

cytosine and uracil were identified chromatographically (Bartnicki-Garcia and Nickerson, 1962a). Ribose in cell wall hydrolysates suggests that nucleic acids may occur in other fungal cell walls.

Melanin-type pigments are present in some fungal walls. Lowry and Sussman (1958) referred to a melanised layer in ascospore walls of Neurospora tetrasperma. A dark pigment extracted from walls of Aureobasidium pullulans has been characterised as melanin on the basis of solubility tests and absorption spectra (Lingappa, Sussman and Bernstein, 1963). Similar material was found in Cladosporium mansoni walls (Sussman, Lingappa and Bernstein, 1963). Structural material in fruiting bodies of Polyporus lispidus may be lignin-like (Bu'Lock, Leeming and Smith, 1962).

PART IV

FUNGICIDES

A. Sites of Reaction

Most protective fungicides have a very low toxicity on a spore weight basis and probably act as non-specific poisons rather than as inhibitors of a particular enzyme group (Somers, 1966). Such materials are generally considered to act by inhibiting spore germination. The distinction between sites of detoxication and sites of toxic reaction is important; active materials may be held or decomposed before reaching vital receptor sites. Copper, however, acts as a fungicide within the protoplast and as a fungistat at the cell surface (Miller, 1969). Possible sites of toxic reaction are at the cell wall, at the cytoplasmic membrane or within the protoplast. The first two sites are considered here.

Although many inorganic and organic toxicants are distributed within fungal spores (Owens and Miller, 1957; Somers, 1963a), Rothstein and Hayes (1956) have shown evidence of the reversible reaction of heavy metals with yeast cell surfaces. The fungicides dodine and glyodin are cationic surface-active agents and by analogy with bactericides they might be expected to cause lysis of the protoplast membrane. Somers and Pring (1966) suggest however that much of the dodine is detoxified by reaction at the cell wall. In common with other micro-organisms fungal spores have a negative charge over a wide pH range (Douglas et al., 1959; Hannan,1961). The nature of these ionogenic surfaces varies from species to species. It is with these surface groups that the initial and possibly toxic reaction with a fungicide - which may be an

ionic species - occurs. Solubility of a fungicide in surface lipid may also be important. The cytoplasmic membrane of a fungal spore is probably similar to that of other cells and consists of a bimolecular leaflet of phospholipid coated with protein (Robertson, 1959). Negatively charged binding sites for heavy metals have been demonstrated at or near the membrane of yeast cells (Passow, Rothstein and Clarkson, 1961). Similar sites in fungal spores may be available for reaction with fungicides.

B. Microelectrophoretic studies of toxicants

Microelectrophoresis has not previously been used to study the site of reaction of fungicides. The method has however been employed in investigations on bacteriostatic and bactericidal agents. Barry and James (1952) showed that the mobility of Aerobacter aerogenes was unaffected by treatment with a toxic dose of formaldehyde thus indicating a lack of surface reaction. In contrast increasing concentrations of proflavine (2:8 di-aminoacridine sulphate) caused a linear decrease in the surface charge of the same organism (James and Barry, 1954). Cells adapted to withstand moderate concentrations of the drug showed different electrokinetic properties than normal cells. Lowick and James (1955) showed increasing concentrations of crystal violet caused a decrease in the mobility of A.aerogenes. Electrophoretic and other measurements showed that cells adapted to crystal violet had a different surface composition from normal cells (Lowick and James, 1957).

The effects of bactericidal surfactants on surface charge have been widely studied. At concentrations much lower than those required to cause lysis and death, surfactants produce

changes in mobility which can be related to the presence of surface lipids (Hill, et al., 1963b). Dyar and Ordal (1946) in studies on various bacteria found that at higher levels, the mobility of cells suspended in sodium dodecyl, tetradecyl or hexadecyl sulphate was either constant or became more negative with increasing concentrations of detergent. Treatment with cetylpyridinium chloride always yielded the same pattern of decrease of charge, reversal and finally stabilisation of positive charge. Using cetyltrimethyl ammonium bromide McQuillen (1950a) found a similar pattern with Escherichia coli, but the charge on Staphylococcus aureus and Streptococcus faecalis became more negative before a positive charge was established. The maximum value was coincident with saturation of the cells with detergent and it was postulated that at this point surface rearrangement or breakdown occurred.

The mode of action of phenolic compounds has been investigated. Haydon (1956) in studies of Escherichia coli showed a correlation between the percentage cells killed by a given phenol concentration and the "mean zeta potential" fall of the cell suspension. The presence of cations gave partial protection against phenol toxicity. The effects of phenol and substituted phenols on the mobilities of young Aerobacter aerogenes cells were measured by James, et al. (1964). An initial drop in mobility at low toxicant level was followed by a sharp rise at higher concentrations. Minimum mobility occurred at the bactericidal concentration. The increase in mobility may be due to the presence of a layer of phenoxy ions held by van der Waals forces to phenol already bound to receptor sites. The compounds had no effect

on the electrophoretic properties of old cells, probably due to the build up of capsular material. Hugo and Franklin (1968) used a homologous series of substituted phenols to test the effect of cellular lipids upon resistance of Staphylococcus aureus. The effects of phenols on the electrophoretic mobilities of high lipid and normal cells were measured. A high surface lipid content may cause "drug trapping" - a process analagous to fungicide detoxication. The microelectrophoretic technique proved more effective than chemical analysis or electron microscopy for detecting drug/cell intractions.

PART V

THE PRESENT INVESTIGATION

Little information is available concerning the chemical composition of fungal spore walls. The spore surface is an important factor in spore dispersal. Protective fungicides act by inhibiting spore germination and vary widely in their effectiveness against different species. The initial reaction with fungicides must be with reactive groups at the spore surface. The purpose of the present study is to investigate the properties of the fungal spore surface since this is important in relation to spore dispersal and reaction with fungicides.

The work is divided into four parts. Part I deals with the determination of pH/mobility curves of spores of a number of fungal species representative of the main classes of fungi. Confirmation of the identities of surface groups is effected using specific reagents. Protoplast surfaces are also investigated. In part II the presence of surface lipids on a wide range of spores is determined and a detailed study made of the surface and wall fatty acid and alkane components of selected species. Part III contains an account of the surface structure and components of Penicillium spores with special reference to Penicillium expansum. The effect of cationic surface-active fungicides on the electrophoretic mobility of spores and of protoplasts is considered in part IV in an attempt to correlate fungitoxicity with reaction at the cell surface.

P A R T I

THE ELECTROKINETIC PROPERTIES OF SOME FUNGAL SPORES

PART 1

THE ELECTROKINETIC PROPERTIES OF SOME FUNGAL SPORES

Little is known of the chemical groups responsible for the wide variations in surface properties of fungal spores. As a preliminary to more detailed examinations a general survey of spores of a wide range of species including several important plant pathogens has been undertaken. Encysted zoospores, sporangia, sporangiospores, conidia, basidiospores, teleutospores and uredospores have been examined. The electrokinetic properties of isolated spore walls and of protoplasts have also been investigated.

Materials and Methods

Fungal Material

Spores of the following species were used

Fungal species	Spore type	Medium
Alternaria tenuis Nees	Conidia	Carrot agar (see Appendix 1).
Botrytis fabae Sardiña	Conidia	as Hislop (1967) (see Appendix 1).
Erysiphe graminis DC. ex Mérat	Conidia	Oat seedlings (var. Black Supreme).
Mucor rouxii (Calmette) Wehm.	Sporangiospores	Tomato juice agar (Haidle & Storck, 1966) (see Appendix 1).
Nectria galligena Bres.	Conidia	Potato carrot agar.
Neurospora crassa Shear & Dodge macroconidial wild type Em 5297a	Conidia	as Richmond & Somers (1962) (see Appendix 1).
Phytophthora infestans (Mont.) de Bary	Sporangia and Zoospores	Sporangia from cultures grown on potato slices (var. King Edward) at 18°. Zoospores liberated by incubating sporangia in distilled water at 2°.
Podosphaera leucotricha (Ellis & Everh.) Salm.	Conidia	Apple leaves (var. Cox's Orange Pippin).

Fungal species	Spore type	Medium
Pseudoperonospora humuli (Miyabe & Takah.) G.W.Wilson	Sporangia	Hop leaves (var. Eastwell Golding).
Puccinia lapsanae Fckl.	Uredospores	Leaves of Lapsana communis L.
Puccinia pruni-spinosae Pers. [= Tranzschelia pruni-spinosae (Pers.) Diet]	Uredospores	Plum leaves (var. Victoria).
Stereum purpureum (Pers. ex Fr.) Fr.	Basidiospores	Fructifications on pear (var.Hendre Huffcap).
Urocystis anemones (Pers.) Winter	Teleutospores	Eranthis hyemalis Salisb. (Winter aconite).
Venturia inaequalis (Cooke) Winter	Conidia	Apple leaves (var. Cox's Orange Pippin).
Verticillium albo-atrum Reinke & Berth.	Conidia	Prune-extract agar (Talboys, 1960) (see Appendix 1).

All organisms grown on agar media were incubated at 25° with the exception of Botrytis fabae which was maintained at 15°.

Alternaria tenuis was isolated from fallen black-currant leaves (Dr.A.J.K.Corke), Botrytis fabae from bean plants (Dr.E.C.Hislop) and Nectria galligena from apple shoots (Mr.V.Jordan), all at Long Ashton Research Station. Phytophthora infestans (Isolate 4DS) and Pseudoperonospora humuli cultures were supplied by the Department of Botany, University of Hull. Verticillium albo-atrum originated from infected hop plants (Dr. P.W.Talboys, East Malling Research Station) and Mucor rouxii and Neurospora crassa were supplied by the Commonwealth Mycological Institute. All other fungi were obtained from naturally infected material at Long Ashton Research Station.

Spores of Alternaria tenuis, Botrytis fabae, Mucor rouxii, Nectria galligena and Neurospora crassa were washed from 7 day cultures with a fine jet of water. Spores from natural material were harvested by a similar technique. Conidia of Podosphaera leucotricha were gently brushed from the infected leaves into aqueous suspension. All the spores were filtered through fine cheese-cloth. Protoplasts were prepared by suspending young hyphae of A.tenuis and N.crassa, and conidia of B.fabae in 0.05M potassium phosphate buffer (pH 6.8) containing 0.58M sucrose and 10^{-3}M glutathione, and incubating with Helix pomatia digestive juice extract (L'industrie Biologique Français, Gennevilliers, France) for 15 hours at 30° (Kinsky, 1962). Protoplasts were filtered through glass wool and finally stabilised in 0.58M sucrose maintained at pH 5.6 with sodium acetate buffer of ionic strength 0.05; at 4° the protoplasts were stable for up to 2 days. Cell walls were obtained by shaking dense spore suspensions at 4° with ballotini (No.12) in a Mickle disintegrator. The centrifuged cell walls, which retained the shape of intact conidia, were washed ten times with 10% (w/v) sucrose, five times with 0.9% (w/v) sodium chloride and five times with water (Dyke 1964). Sporangiospores of Mucor rouxii were converted to the yeast-like form by incubation at 25° for 18 hours under nitrogen in the defined medium of Bartnicki-Garcia and Nickerson (1962b) containing 0.2% vitamin-free casein hydrolysate as nitrogen source (see Appendix 1). Yeast-like cells could be converted to the filamentous form by aerobic incubation for 4 hours (Haidle and Storck, 1966).

Microelectrophoresis

Electrophoretic mobilities of conidia, cell walls, and

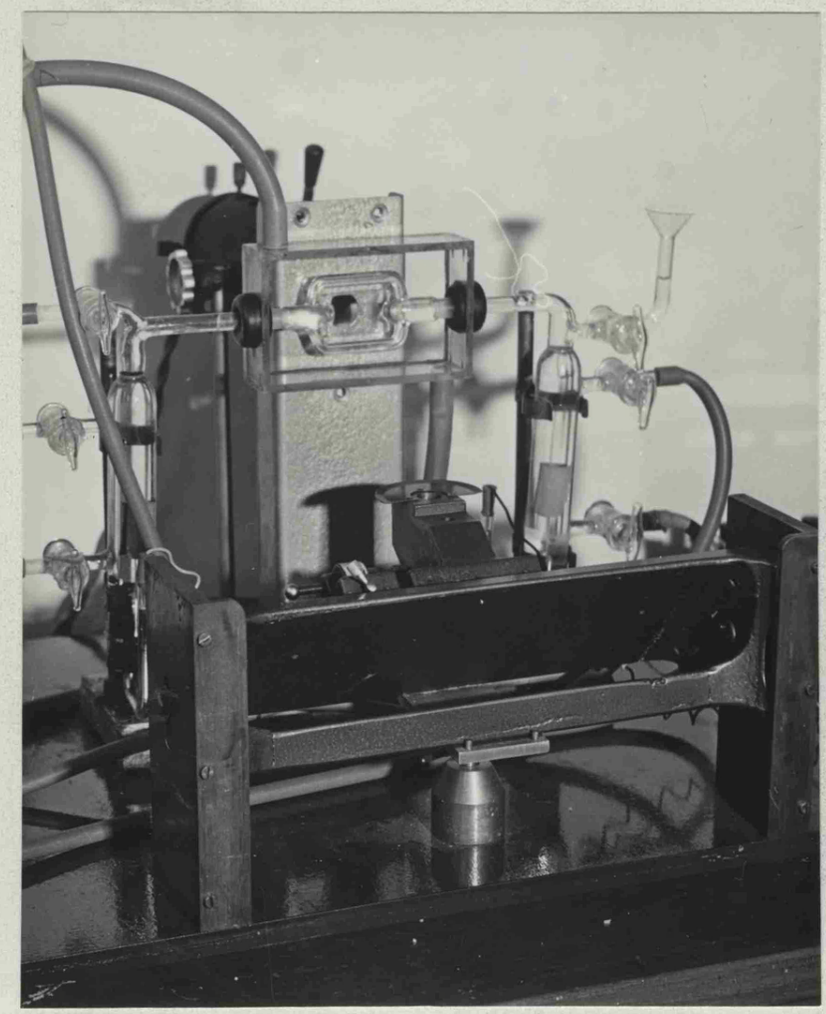


Plate 1. Apparatus for microelectrophoresis showing the water jacketed cell and the electrode arrangement. Dial gauge and microscope removed.

protoplasts - all usually at a concentration of 1 million/ml - were measured in a rectangular closed cell enclosed in a water jacket at 25° ± 0.2° (Plates 1 and 2). The apparatus was a modification of that described by Gittens and James (1960), the cell being mounted in the lateral position (Hartman et al., 1952) and the X 20 water-immersion objective focussed on the stationary layer through a close fitting rubber sheet. The microscope incorporated a X 10 magnification eyepiece containing a calibrated cross-hatched graticule. Focussing

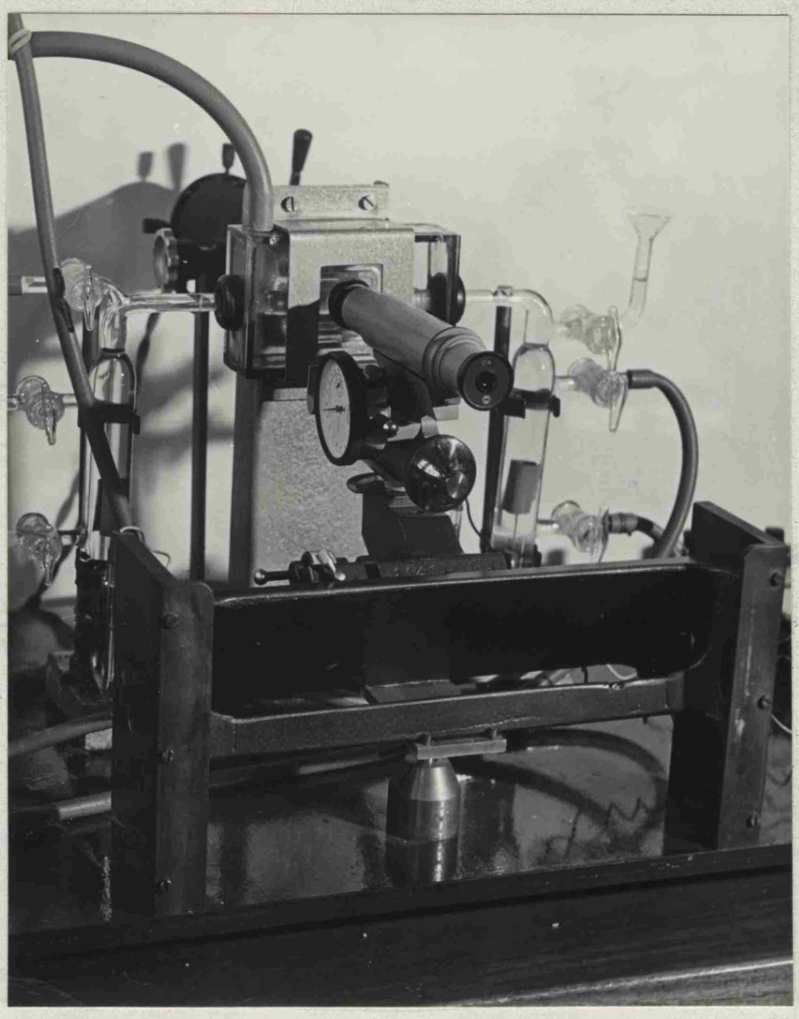


Plate 2. Apparatus for microelectrophoresis showing the microscope and dial guage in position.

at the stationary levels was effected by measuring movement of the microscope using a dial guage. A variable-intensity microscope lamp provided illumination. Particle velocity was measured over 180 μ in both directions (current reversal) and each mobility was obtained from at least 20 observations: the standard error of the mean was less than 4%. Appendix 2 shows statistical analysis of a typical set of readings. Silver/silver chloride electrodes were used (Figure 3). The circuit diagram is shown in Figure 4. A "Shandon" stabilised D.C.power supply replaced the dry batteries in later experiments. The conductivity of the buffered particle suspensions was measured

in a stoppered conductivity cell at 25° using a Wayne-Kerr B221 bridge.

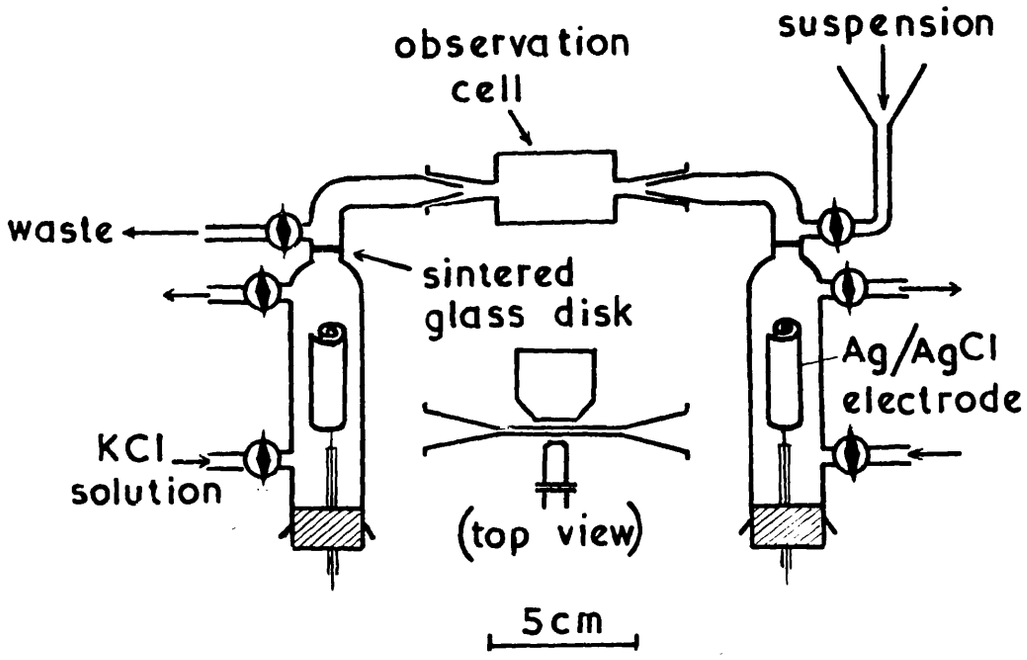


Figure 3. Arrangement of electrodes and observation cell.

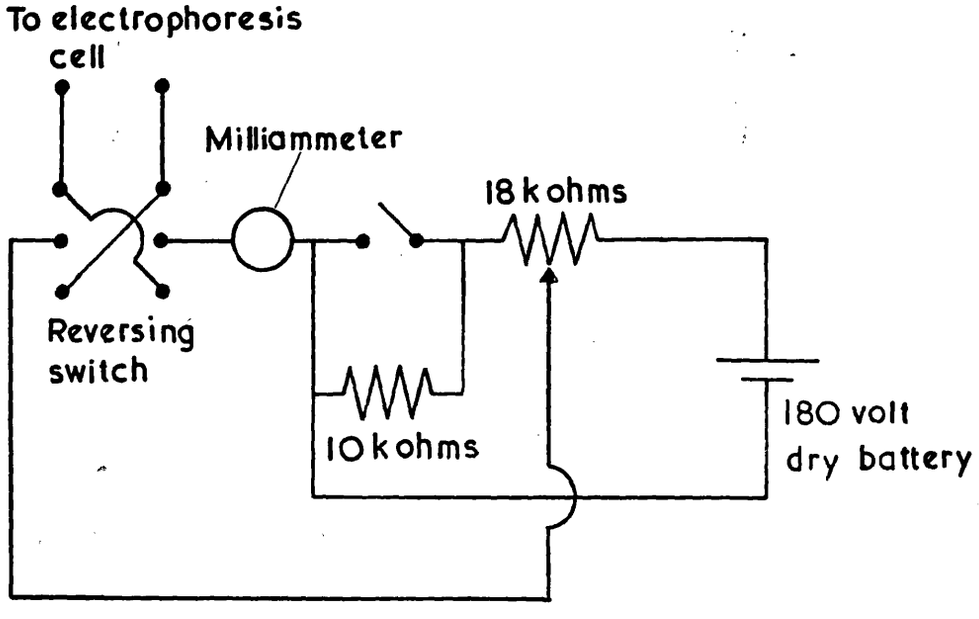


Figure 4. Circuit diagram.

Calculation of electrophoretic mobilities

The electrophoretic mobilitiy of a particle is defined as the velocity per unit field strength

$$\bar{V} = \frac{V}{X} \qquad \ldots\ldots\ldots \quad \text{(Equation 2)}$$

where $\bar{V}$ is the electrophoretic mobility (μ/sec/volt/cm), V the electrophoretic velocity (μ/sec) and X the field strength (volts/cm). It is therefore necessary to determine both the velocity of a particle and the field strength producing that movement. The field strength may be calculated by measuring the current (i amps) flowing through the cell and applying Ohm's law i.e.

$$X = \frac{i}{AK} \qquad \ldots\ldots\ldots \quad \text{(Equation 3)}$$

where A is the cross sectional area (sq.cm) and K the specific conductivity of the suspension (mhos).

Calibration of Electrophoresis Cell

Although it is difficult to determine the internal dimensions of a fused rectangular cell by direct measurement, the cross-sectional area may be readily calculated by measuring the velocity of standard particles at the stationary levels. A suspension of human red blood cells in 0.067M phosphate buffer (pH 7.4) at 25°, shown to have a mobility of 1.31 μ/sec/volt/cm (Abramson, 1929; Seaman, 1965), provides a suitable standard.

The specific conductivity of .067M phosphate was found to be .0087 mhos at 25°, and with a current flow of 8.00 mA the mean particle velocity (20 readings in each direction) at the stationary level was 13.15 μ/sec. From Equation 2 the field strength (X) is equal to 10.04 volts/cm. Substituting

this value in Equation 3 the cross sectional area (A) was calculated as 9.15 mm^2.

Check on Cell Symmetry

Cell symmetry was tested by determining the velocity of conidia of Neurospora crassa at different depths. The data conform closely to a parabolic velocity profile (Figure 5).

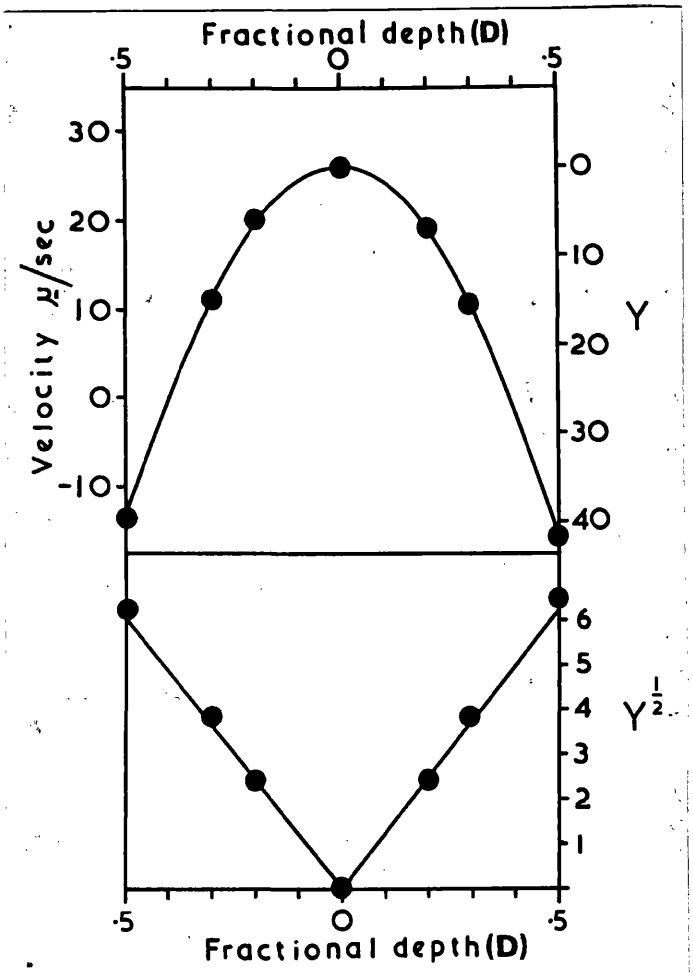


Figure 5. Velocity profile for Neurospora crassa as a measure of cell symmetry. Abscissa:Fractional distance from centre of cell to wall. Ordinates:Upper left, actual velocities of migration. Upper right, difference Y between velocity of migration at midpoint of the cell and the velocity of migration at depth D. Lower right, square root of Y.

Suspension media

Conidia, cell walls, and protoplasts were washed twice with the buffer to be used in mobility measurements before adding to the electrophoresis cell. All buffer solutions used were of a final ionic strength of 0.05. The following buffer solutions were used for pH/mobility curves (Gittens and James, 1963); below pH 2.6, HCl and NaCl; pH 2.6 - 9.6, NaCl and sodium acetate and sodium barbiturate and HCl; above pH 9.6, NaCl and sodium acetate and sodium barbiturate and NaOH. Measurements of protoplast mobilities were made in buffers containing 0.58M sucrose.

Treatments to modify surface groups

Alkaline phosphatase: (EC 3.1.3.1.) Washed conidia and cell walls were suspended in barbiturate buffer (pH 7.9,I:0.02) containing 5 µg alkaline phosphatase per ml for 1 hour at 37° (Hill et al., 1963a).

Diazomethane (DAM). Diazomethane was prepared from N-methyl-N-nitroso-p-toluenesulphonamide (De Boer and Backer, 1954). Washed cell walls were dried in vacuo over phosphorus pentoxide and then suspended for 3 hours in an ether:methanol (1:30 v/v) solution containing excess diazomethane (Best and Durham, 1965). Methylated and control cell walls were incorporated into KBr disks and infrared spectra recorded on a Perkin Elmer 237 spectrophotometer. Conidia were washed once with phosphate buffer (pH 7.0,I:0.05) and twice with HCl (0.05M) before methylation. Washed cells were suspended in an 0.02M solution of diazomethane in ether:ethanol (1:5, v/v) for 15 minutes at room temperature (Gittens and James, 1963). Excess reagent was indicated by the yellow colour of the supernatant.

After methylation cells were washed once in ethanol and twice in the buffer solution to be used for electrophoretic measurements.

1-Fluoro-2,4-dinitrobenzene (FDNB). Conidia and cell walls were washed three times in phosphate buffer (pH 7.0,I:0.05) then suspended in an 0.1% (v/v) ethanolic FDNB solution containing 0.9% (w/v) $NaHCO_3$ for 5 hours (Gittens and James, 1963). The sediment was washed five times with ethanol before washing with the final buffer solution.

Identification of free amino groups

Conidia after treatment with FDNB were hydrolysed in a sealed tube at 105° for 16 hours in the presence of 5.7M HCl. The contents of the tube were cooled, diluted, and extracted with ether. Ether-soluble dinitrophenylamino acids were absent from all the spores examined. The aqueous phase was evaporated to dryness to remove HCl and the residue redissolved in water and passed down a column of "Hyflo-Super-Cel" and talc (28.5:71.5, w/w) to remove free amino acids (Biserte, Holleman, Holleman-Dehove and Sautière, 1960). Dinitrophenylamino acids were identified chromatographically on paper by the method of Ingram and Salton (1957) using butanol: acetic acid: water (3:1:1, by vol.), and on a thin-layer of silica gel by the two dimensional technique of Pataki (1967) using phenol: 0.22M ammonium hydroxide (80:22,w/v) and 2-chloroethanol: toluene: pyridine: 2.86M ammonium hydroxide (50:35:15:10, by vol.). Separations were also carried out on silica gel in a single dimension using n-propanol: 4.86M ammonium hydroxide (70:30, v/v) and the identity of the dinitrophenylamino acids confirmed by the colourations produced on spraying with ninhydrin (Brenner, Niederwiesser and Pataki, 1969).

Electron microscopy

Conidia and cell walls were fixed in 2% aqueous $KMnO_4$ for 1 hour at room temperature, dehydrated through a graded acetone series and embedded in Vestapol. Sections were cut on an LKB Ultratome and stained with lead citrate (Reynolds, 1963). Protoplasts were fixed with 2% $KMnO_4$ in 0.6M KCl (Nečas and Havelková, 1966). Sections of conidia, walls and protoplasts were all viewed using an AEI EM6B microscope. For scanning electron microscopy spores were attached to the metal specimen holders with "Durafix" adhesive. After coating under vacuum with gold/palladium the specimens were viewed in the Stereoscan electron microscope.

Illustrations of some of the spores used in these investigations are shown in plates 3-13.

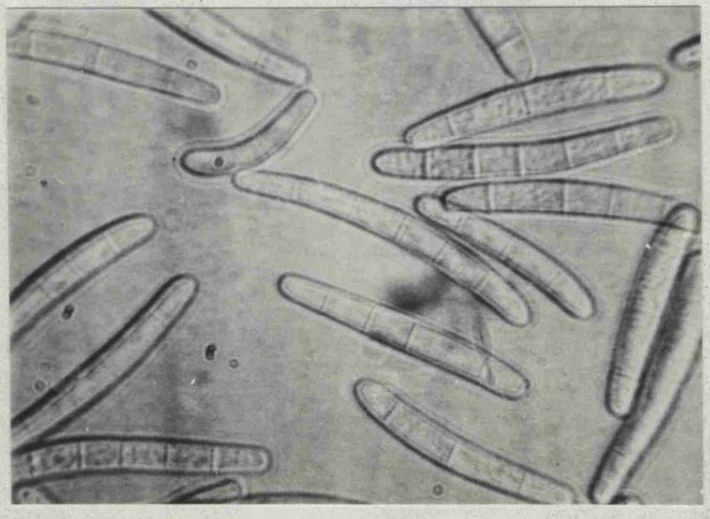


Plate 3. Nectria galligena
conidia X 760.

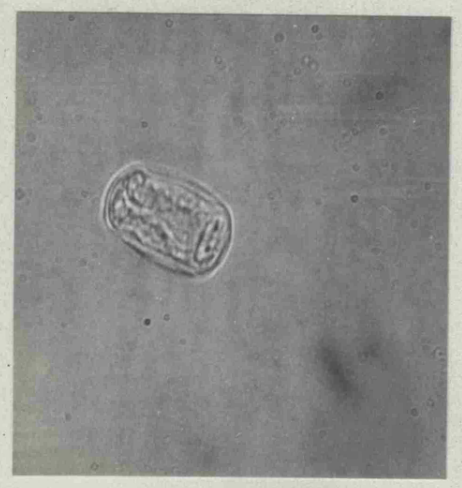


Plate 4. Podosphaera leucotricha conidia X 760.

Plate 5. Venturia inaequalis conidia X 760.

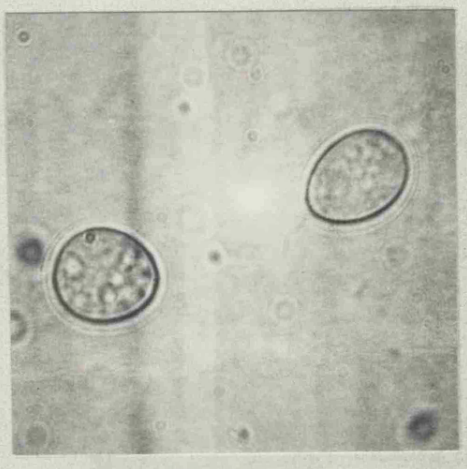


Plate 6. Botrytis fabae conidia X 1000.

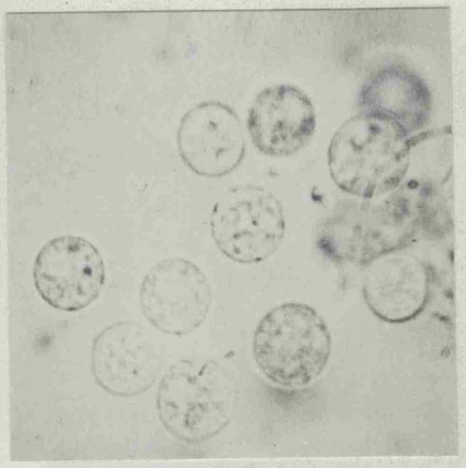


Plate 7. Phytophthora infestans encysted zoospores X 1000.

Plate 8. Phytophthora infestans sporangia X 1000.

Plate 9. Alternaria tenuis conidia X 1000.

Plate 10. Alternaria tenuis conidia X 2500 (Stereoscan).

Plate 11. Puccinia lapsanae uredospore X 3000 (Stereoscan).

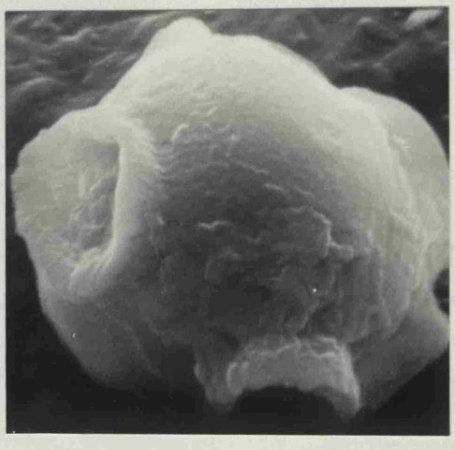


Plate 12. Urocystis anemones teleutospore X 2000 (Stereoscan).

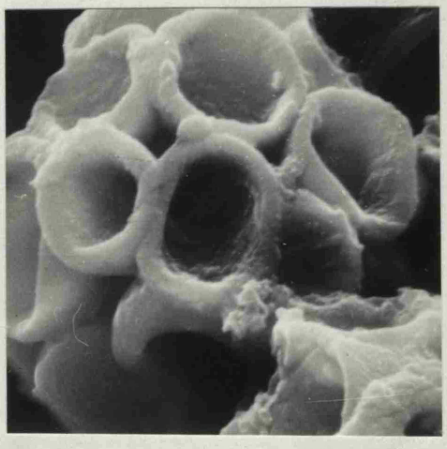


Plate 13. Urocystis anemones teleutospore X 2000 (Stereoscan).

(Plates 12 and 13 illustrate variations in the number of collapsed sterile cells attached to the teleutospore).

Results

Sporangia of Phytophthora infestans (Figure 6) had zero mobility over the whole pH range, indicating the absence of surface ionogenic groups. Encysted zoospores of P.infestans (Figure 6), basidiospores of Sterium purpureum (Figure 7), teleutospores of Urocystis anemones (Figure 8), uredospores of Puccinia lapsanae (Figure 9) and conidia of Nectria galligena (Figure 10) and of Verticillium albo-atrum (Figure 11) showed no positive mobility at low pH. Cells of S.purpureum treated with alkaline phosphatase had a similar mobility to untreated spores, confirming the absence of phosphate groups. Treatment with diazomethane (Figure 7) decreased the

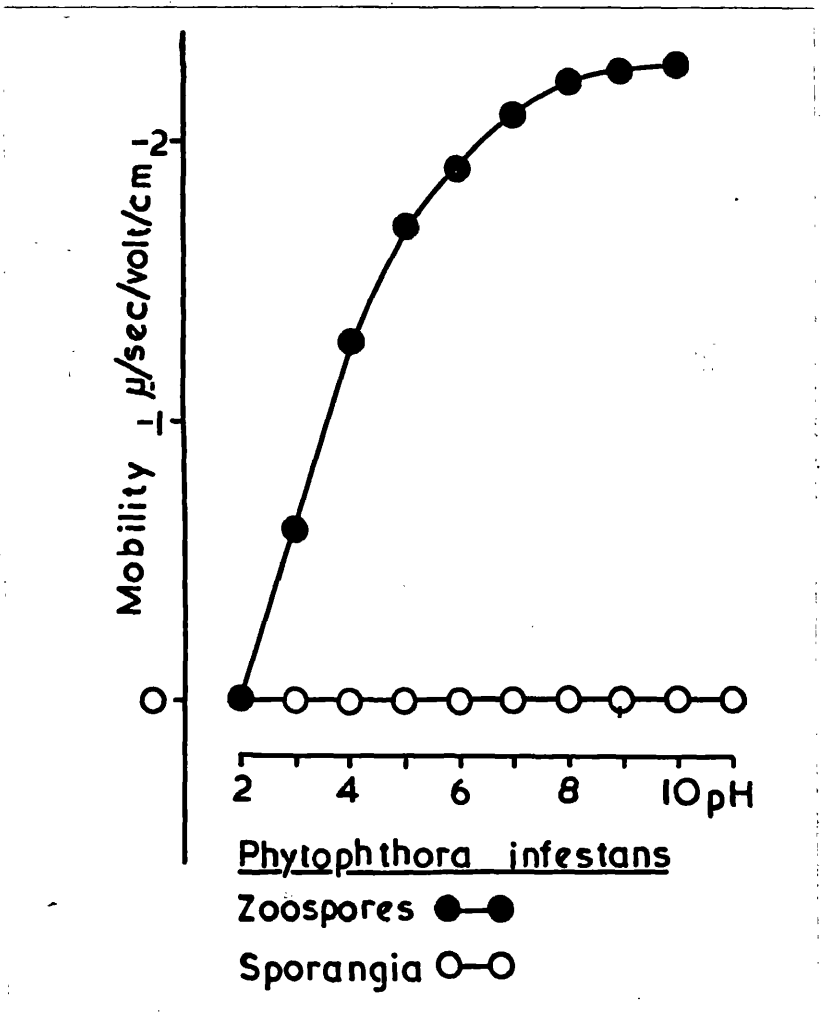


Figure 6. pH/mobility curves of Phytophthora infestans.

mobility to zero between pH 2 and 5, showing that only carboxyl groups contribute to the surface charge. Above pH 7, however, the mobility did not return to its original value after hydrolysis of the methyl esters, suggesting that DAM treatment may block other surface groups. Control experiments showed that suspension in 0.05M HCl followed by ether and ethanol as required for the chemical treatment did not cause irreversible changes to the spore surface. After resuspension in pH 7 buffer mobilities were the same as for untreated spores. Conidia of Podosphaera leucotricha (Figure 12), Erysiphe graminis (Figure 12) and Venturia inaequalis (Figure 7); uredospores of Puccinia pruni-spinosae (Figure 9) and sporangia of Pseudoperonospora humuli (Figure 13) all have high positive values on acid solution, characteristic of mixed amino-carboxyl surfaces (James and List, 1966).

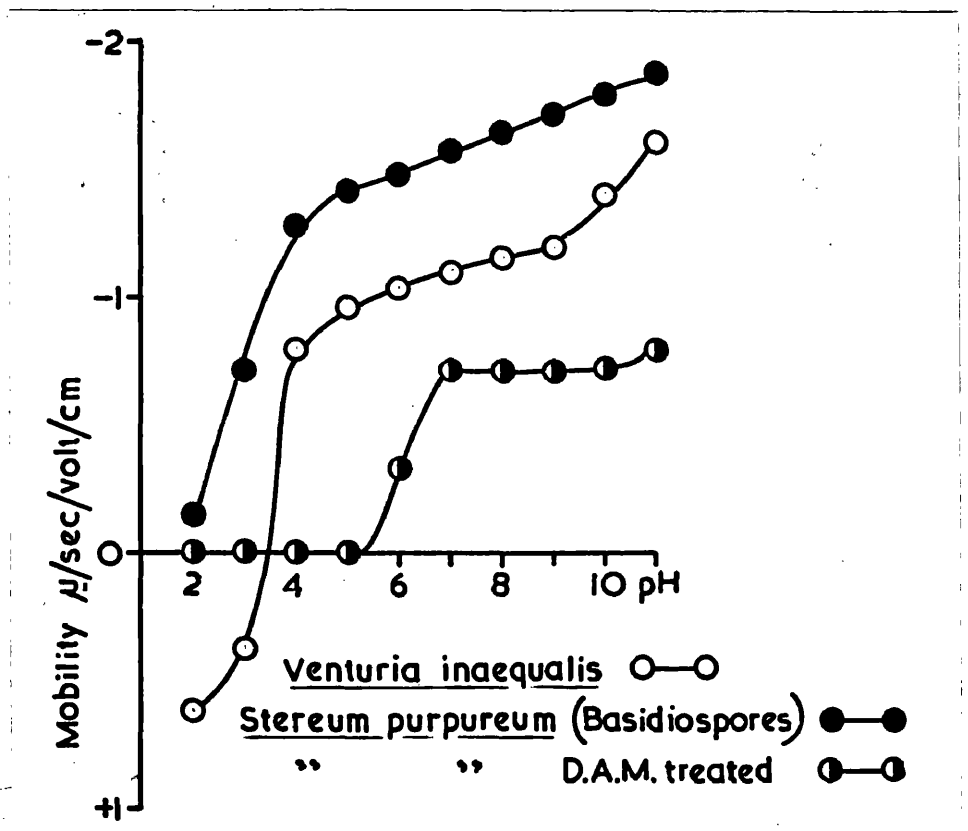


Figure 7. pH/mobility curves of conidia of Venturia inaequalis and basidiospores of Stereum purpureum.

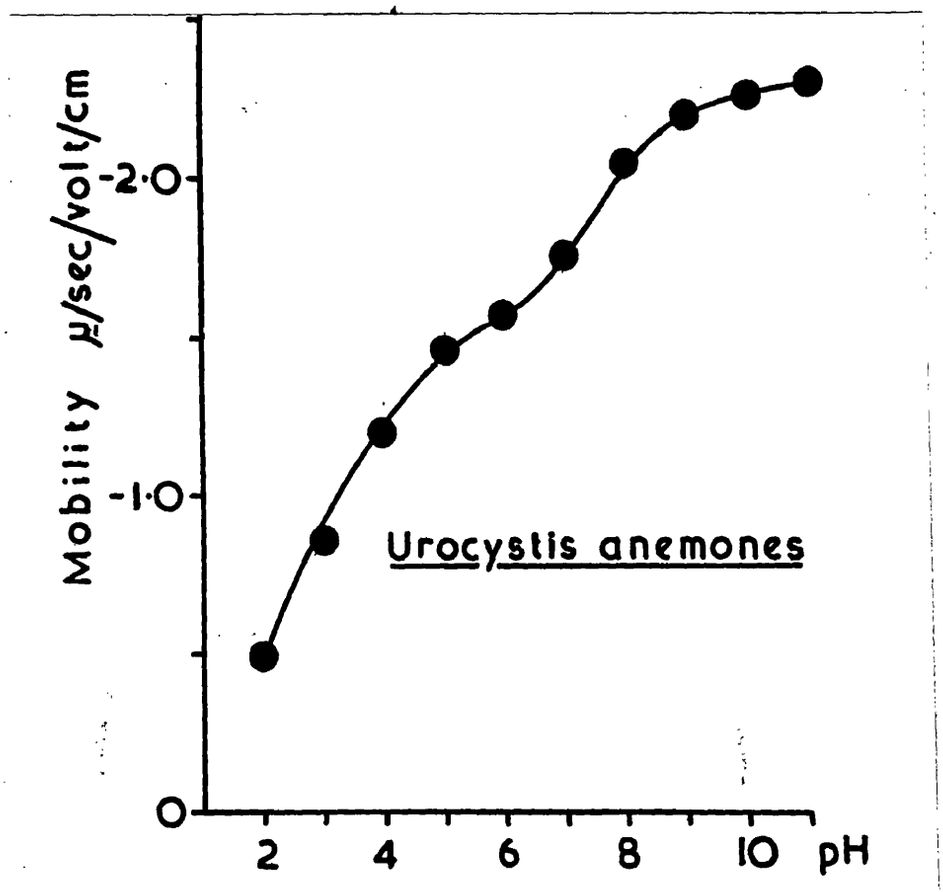


Figure 8. pH/mobility curve of teleutospores of Urocystis anemones.

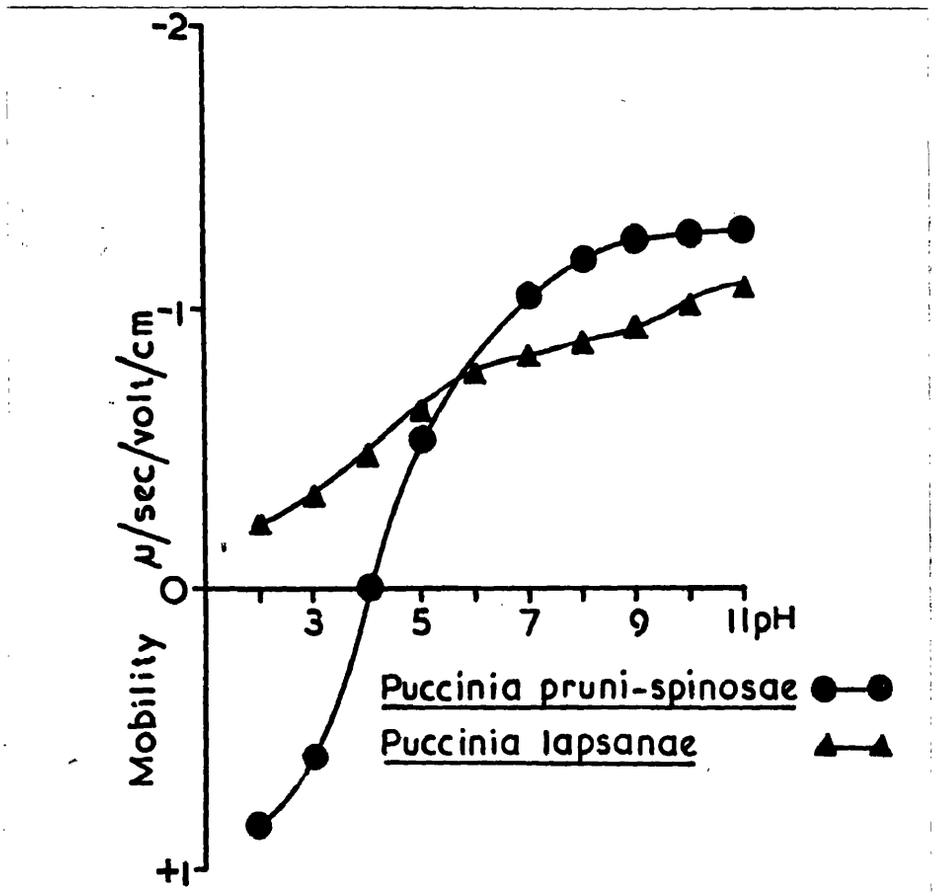


Figure 9. pH/mobility curves of uredospores of Puccinia pruni-spinosae (= Tranzschelia pruni-spinosae) and Puccinia lapsanae.

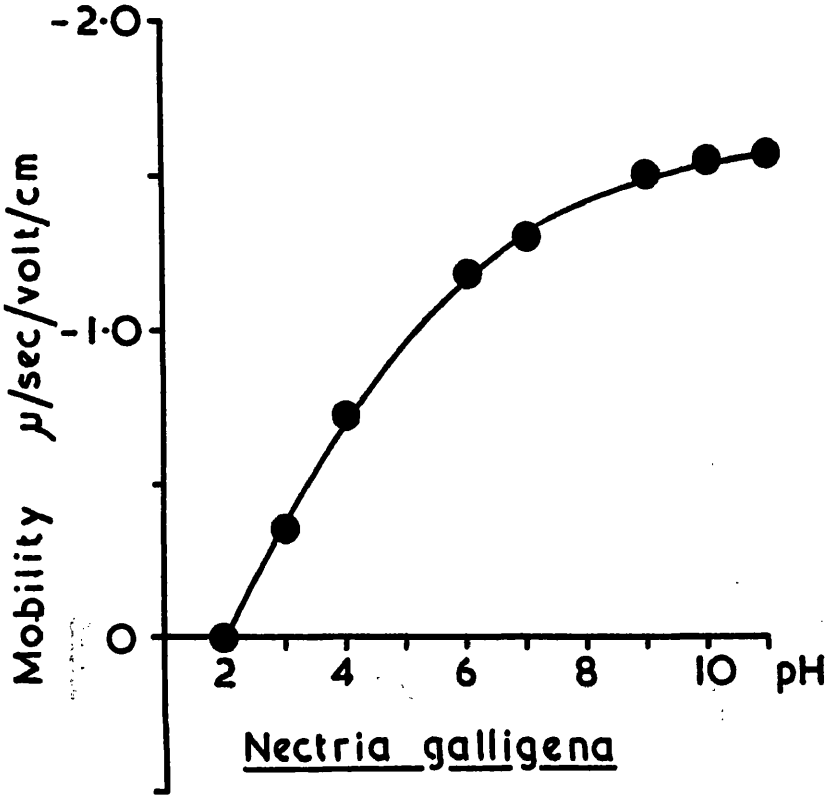


Figure 10. pH/mobility curve of conidia of Nectria galligena.

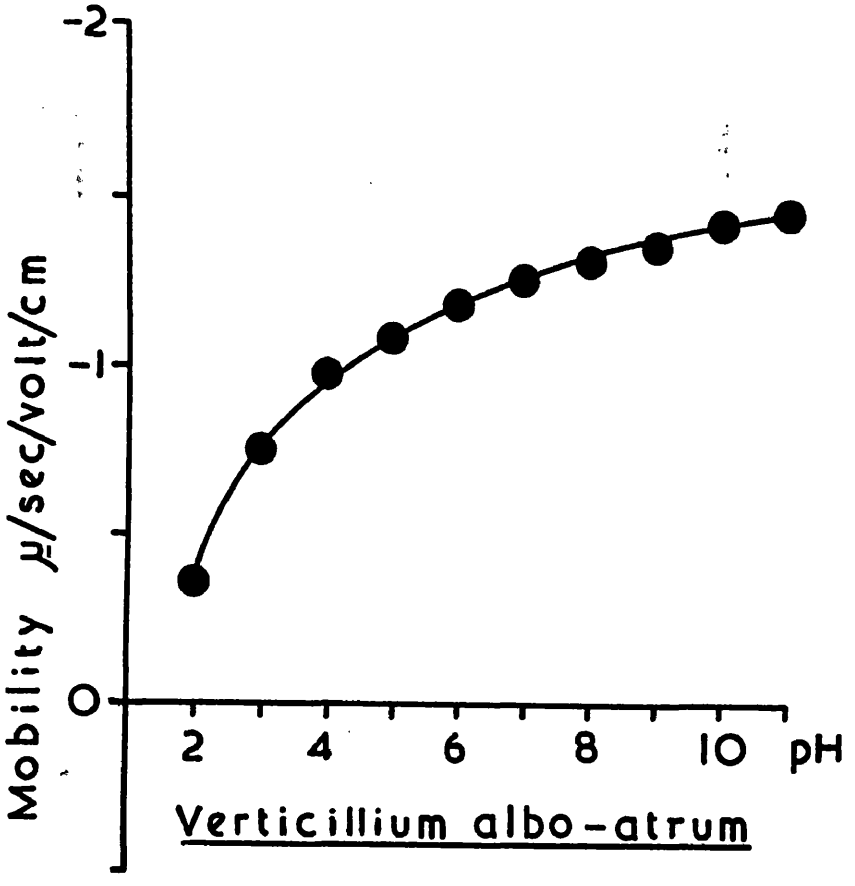


Figure 11. pH/mobility curve of conidia of Verticillium albo-atrum.

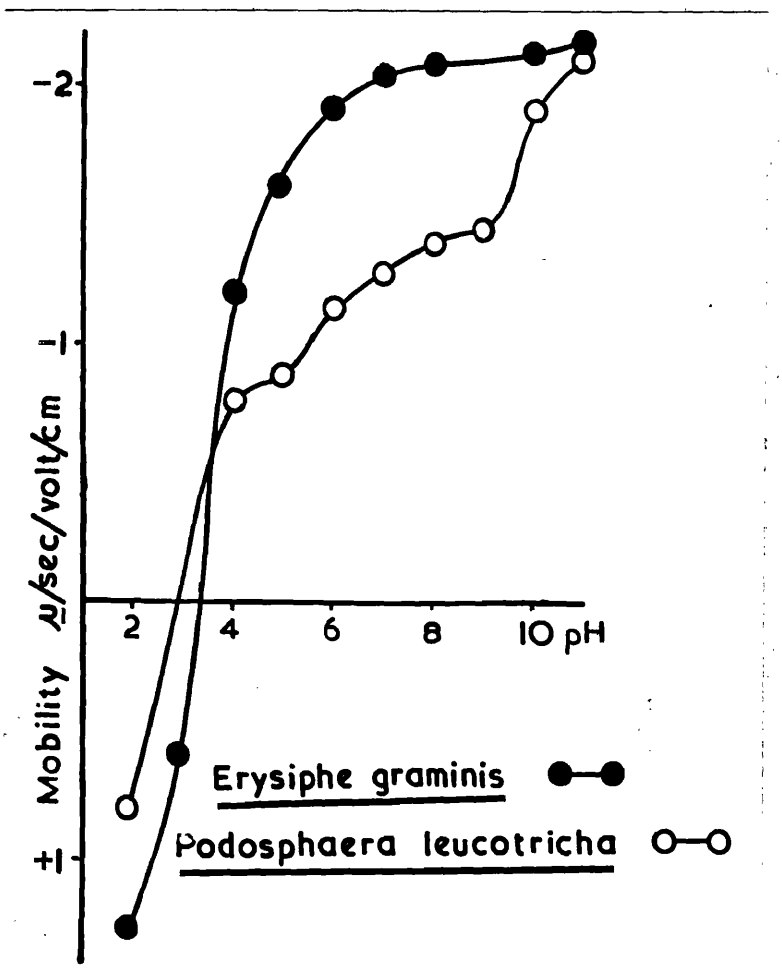
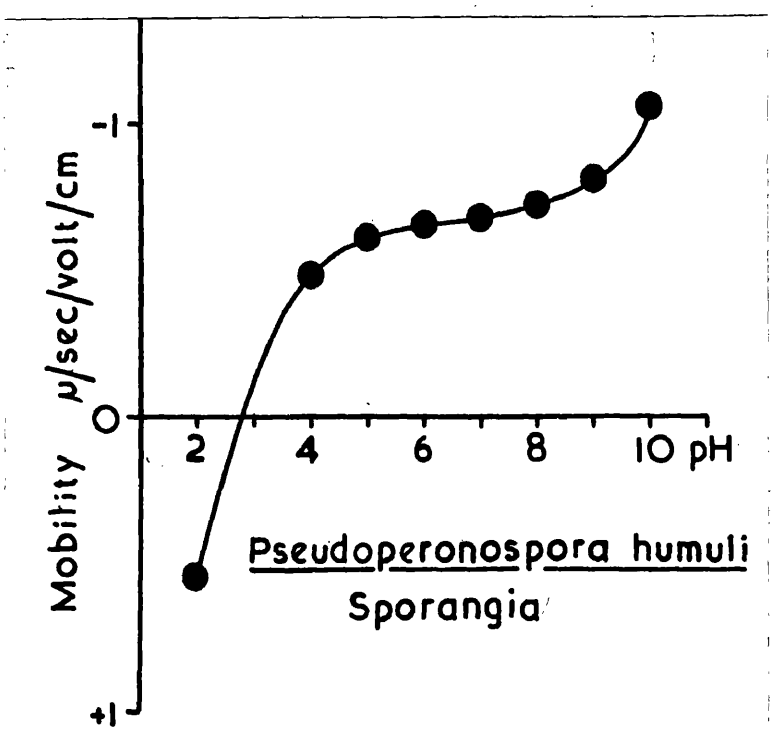


Figure 12. pH/mobility curves of conidia of Erysiphe graminis and Podosphaera leucotricha.



.Figure 13. pH/mobility curve of sporangia of Pseudoperonospora humuli.

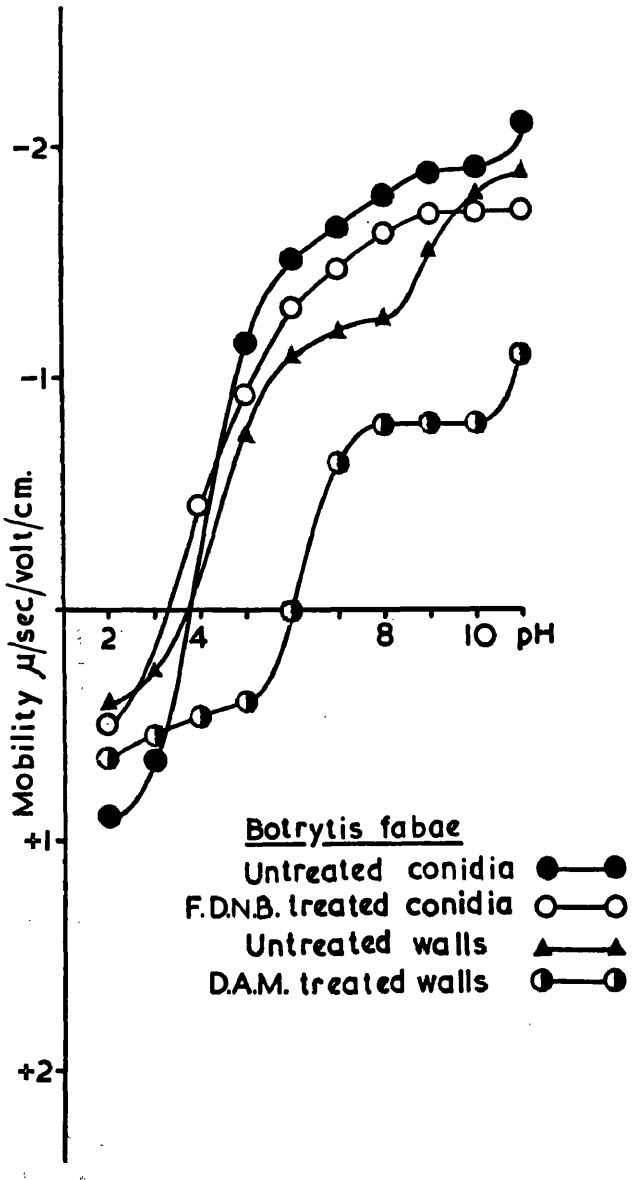


Figure 14. pH/mobility curves of Botrytis fabae.

Treatment of spores of Botrytis fabae (Figure 14) and Alternaria tenuis (Figure 15) with 1-fluoro-2,4-dinitrobenzene decreased the positive mobility at low pH and removed the inflection at pH 10.0, confirming the presence of amino groups on the untreated surface. Hydrolysis of FDNB-treated B.fabae conidia followed by chromatography revealed spots corresponding to the dinitrophenyl derivatives of ε-lysine, histidine, leucine and an unidentified compound; A.tenuis

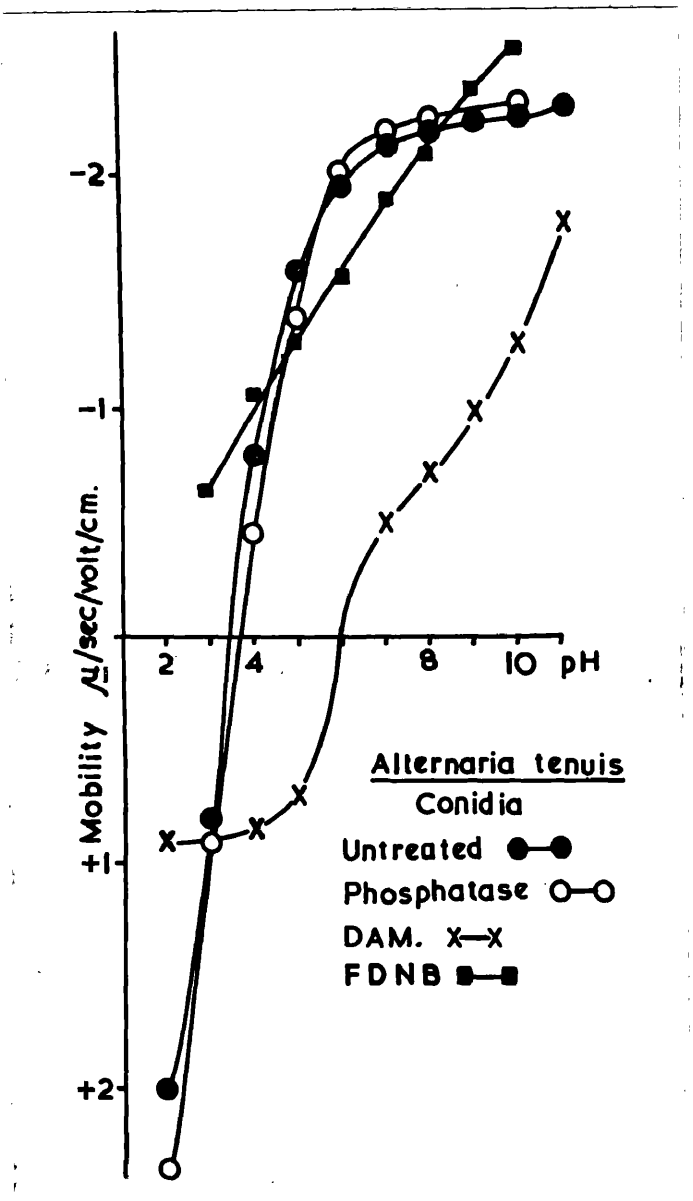


Figure 15. Alternaria tenuis: pH/mobility curves of untreated conidia and of conidia modified by chemical or enzymic treatment.

conidia revealed, in addition, a spot corresponding to DNP-tyrosine. The unidentified compound had a similar R_F value to the DNP derivatives of D-glucosamine and D-galactosamine, and might have been derived from a glucosamine or galactosamine polymer in the cell wall.

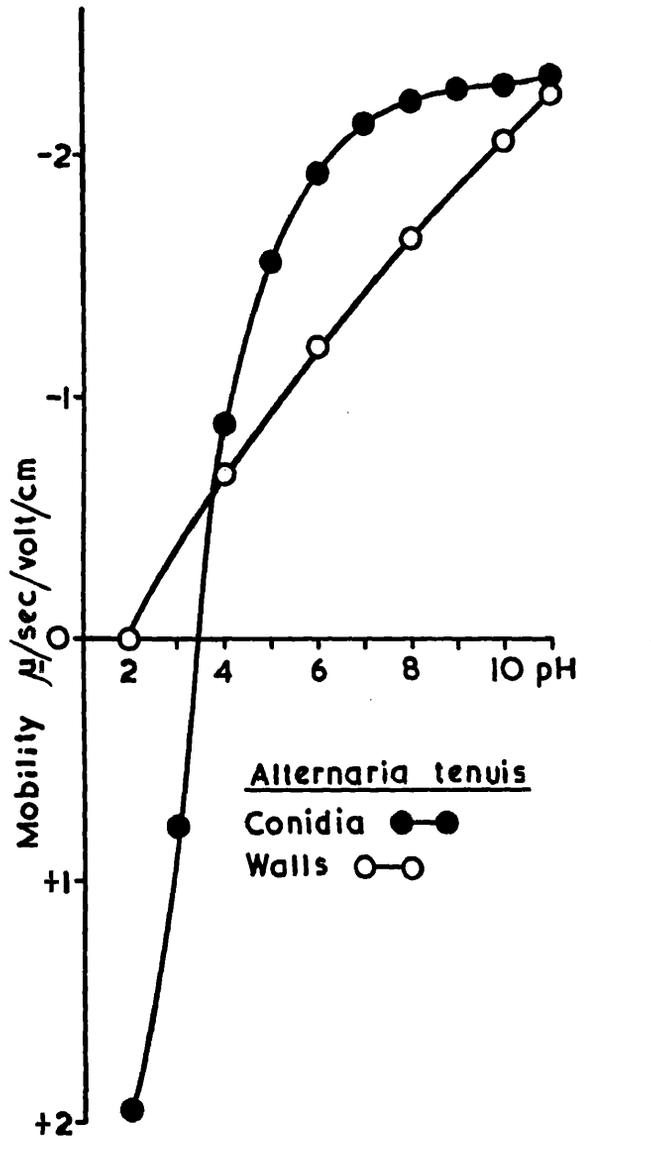


Figure 16. Comparison of pH/mobility curves of conidia and conidial walls of Alternaria tenuis.

Cell walls of Botrytis fabae gave a pH/mobility curve with the same shape and isopotential point (3.8) as that for intact conidia (Figure 14). The curve for A.tenuis conidia was different from that of the conidial walls (Figure 16). The possibility of the removal of a surface layer of mucilaginous material during wall preparation was investigated by electron microscopy, but no differences between the outer surfaces of intact conidia and washed walls were apparent (Plates 14-17). During the preparation of purified

Key to symbols used on plates 14-17: PW - primary wall; SW - secondary wall; SP - septal partition; N - nucleus, M - mitochondrion; V - vacuole

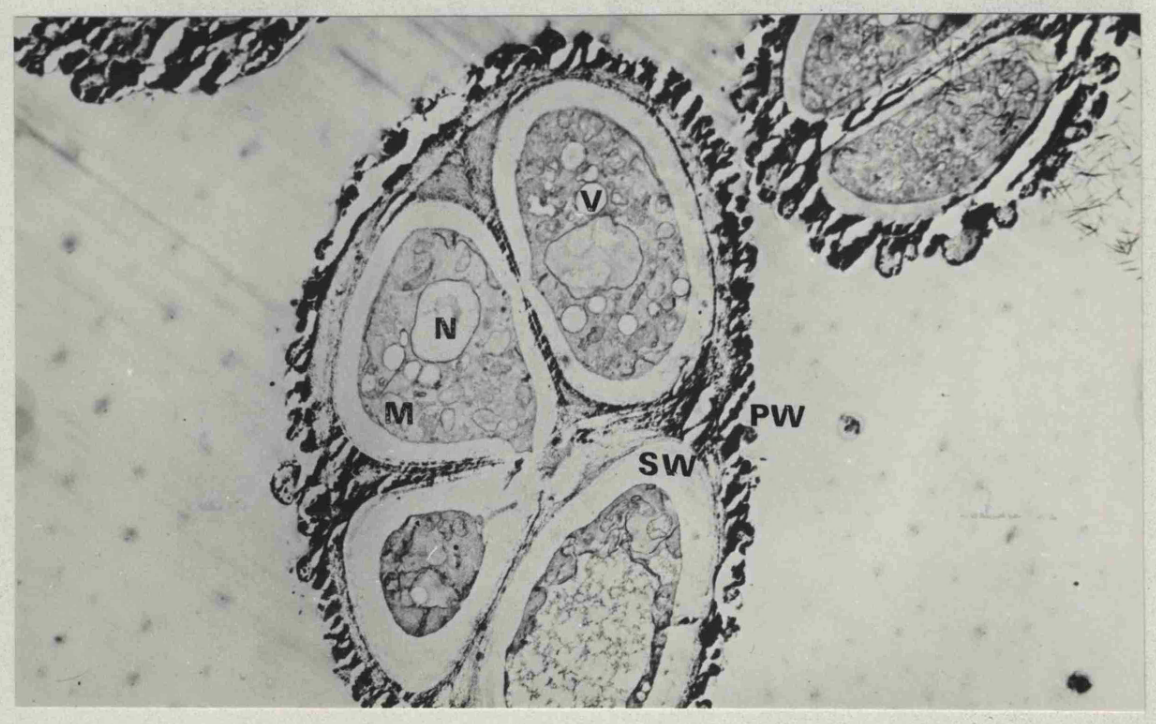


Plate 14. Section of Alternaria tenuis conidium X 6000.

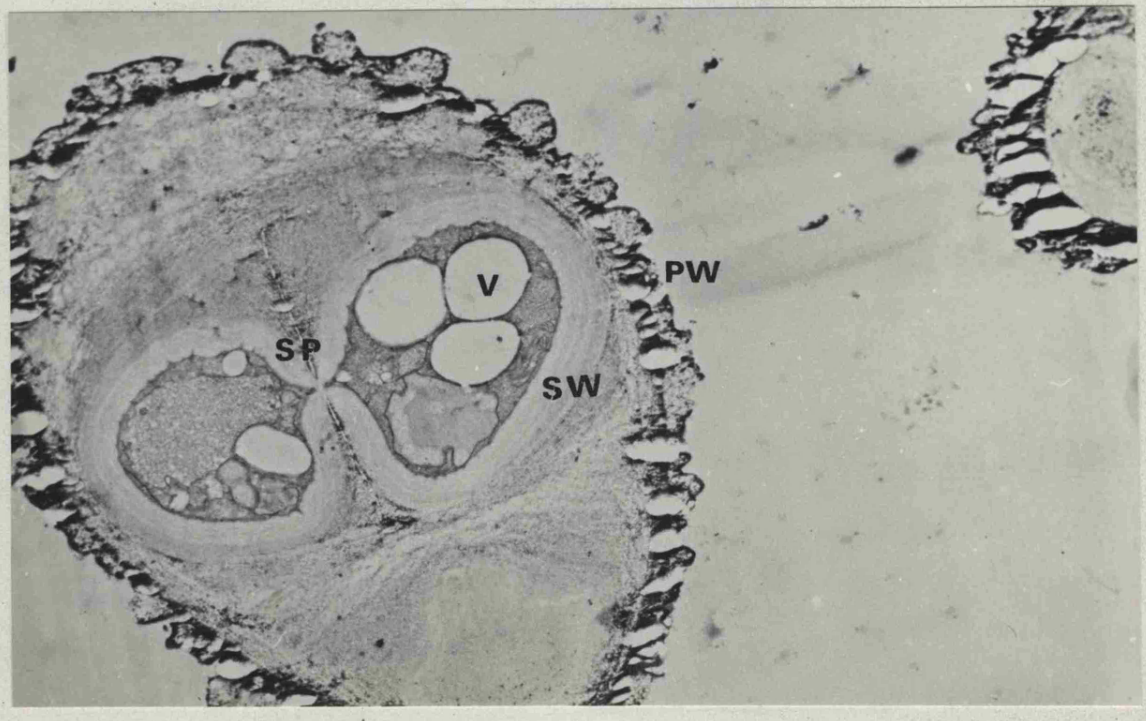


Plate 15. Section of Alternaria tenuis conidium X 8000.

Plate 16. Section of Alternaria tenuis spore wall X 6000.

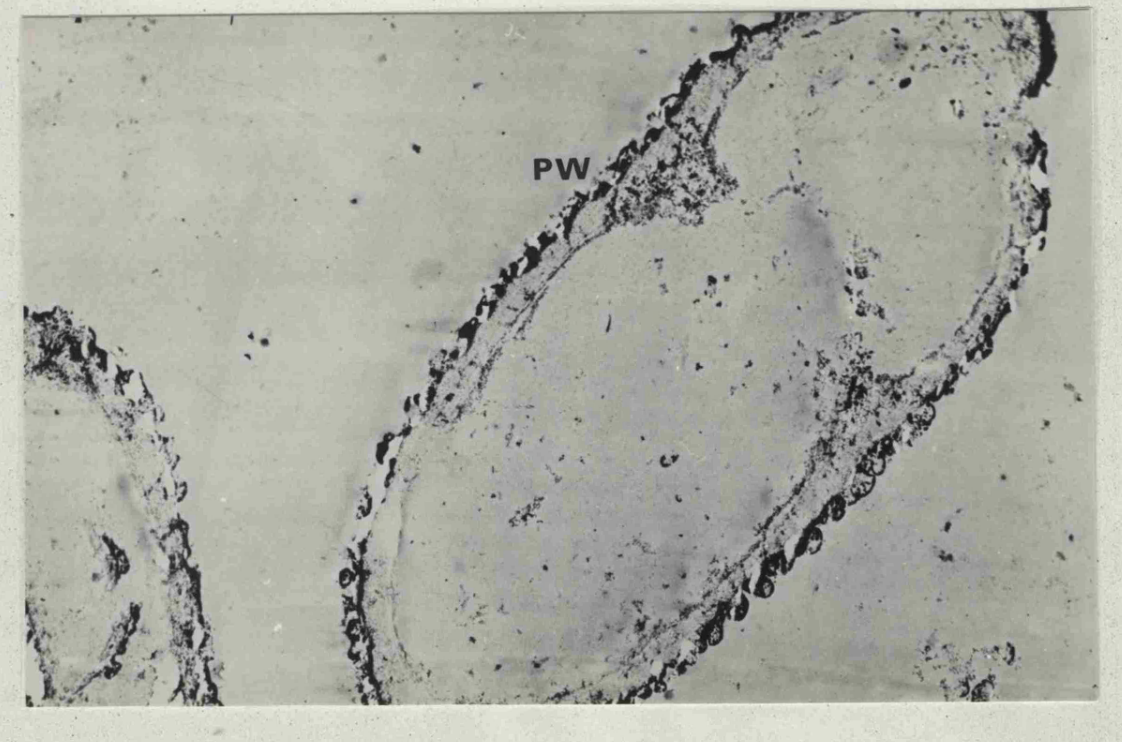


Plate 17. Section of Alternaria tenuis spore wall X 8000.

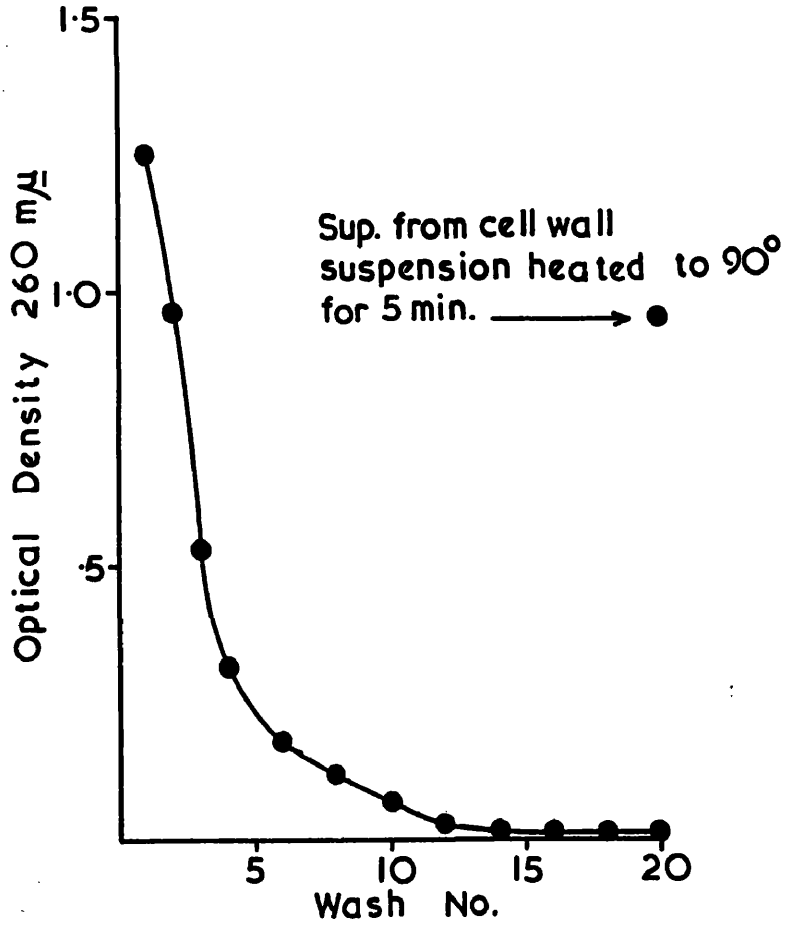


Figure 17. Preparation of purified Alternaria tenuis spore walls. Optical density of wall washings as a measure of cytoplasmic contamination.

spore walls optical density measurements at 260 mμ showed the supernatant to be free of cytoplasmic contaminants after 20 washes by the technique of Dyke (1964). Heating the walls to 90° for 5 minutes in water liberated further nucleic acids however (Figure 17). Although Dyke's technique has been shown to be satisfactory in the preparation of purified cell walls of unicellular spores it is evidently less effective with the multicellular A.tenuis conidia.

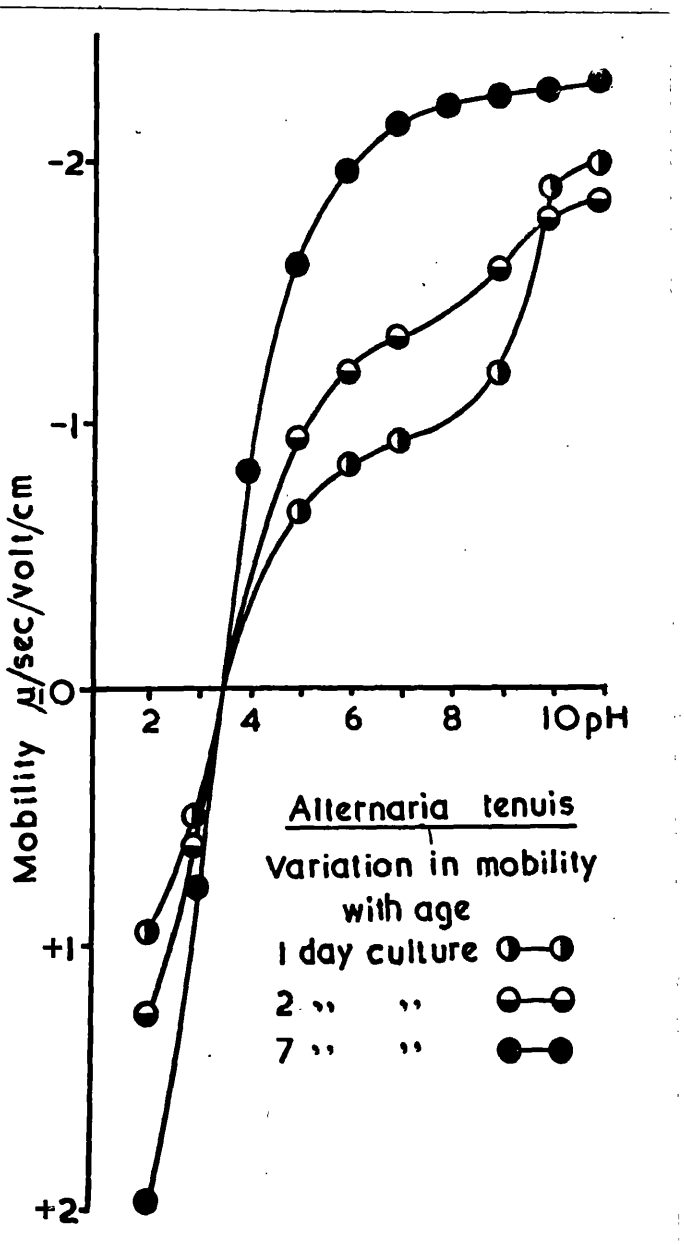


Figure 18. Comparison of pH/mobility curves of conidia of Alternaria tenuis from cultures of different ages.

A comparison of the pH/mobility curves of A. tenuis conidia from cultures of different ages is shown in Figure 18. All curves have the same isopotential value and conform to a mixed amino-carboxyl surface, though the charge density increases as the spores age.

The pH/mobility curve for Neurospora crassa conidia (Figure 19) suggests the presence of strongly acidic groups and free amino groups. Phosphate groups are indicated by an isopotential point as low as pH 2.6 and confirmed by displacement of the curve when conidia were treated with alkaline phosphatase. Mobility of N.crassa conidia towards the anode increased after treatment with FDNB, which is consistent with the blocking of amino groups. After hydrolysis of the treated conidia the dinitrophenyl derivatives of ε-lysine and histidine were identified chromatographically. The surface of washed cell walls of N.crassa differed from that of intact conidia (Figure 20). Unlike the cell surface there was no evidence that phosphate groups of pK 2-3 were present on the wall and treatment with alkaline phosphatase did not alter the isopotential point from pH 4. The more marked increase in mobility of FDNB treated walls compared with similarly treated whole cells suggests an increased proportion of free amino groups. Confirmation of the presence of carboxyl groups on the surface of cell walls was provided by the infrared spectrum of cell walls esterified with diazomethane. Figure 21 shows a pronounced shoulder at 1740 cm^{-1} for treated cells (curve A) due to the carbonyl stretch of the ester: no such absorption was shown by cell walls subjected to the same preparative treatment (i.e. stirred in ether-methanol for 3 hours), but omitting diazomethane (curve B).

The major polysaccharide component of the sporangiospore wall of Mucor rouxii has been shown to be a neutral glucan (Bartnicki-Garcia and Reyes, 1964). Chitosan, the non-acetylated glucosamine polymer, forms the major component of the wall of

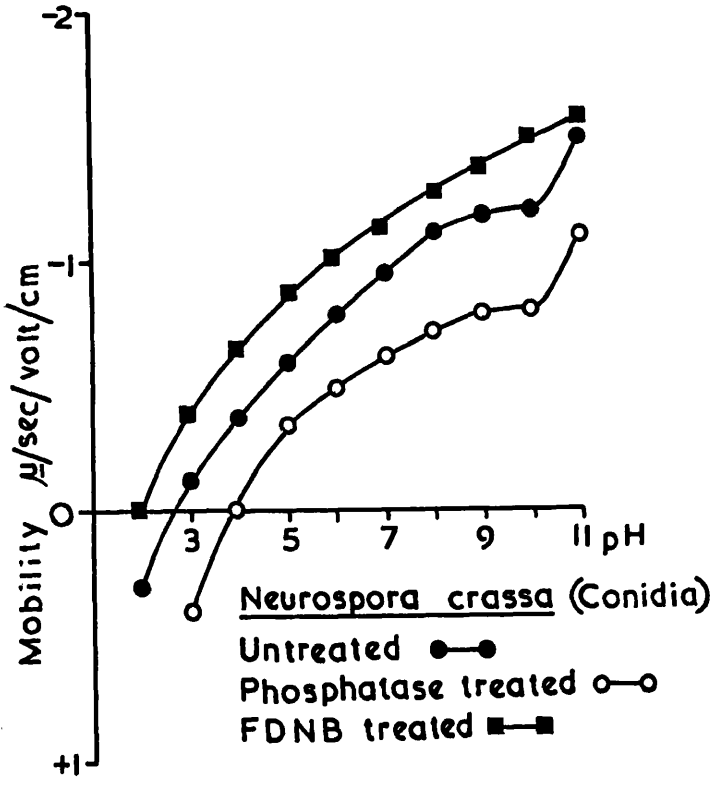


Figure 19. pH/mobility curves of conidia of Neurospora crassa.

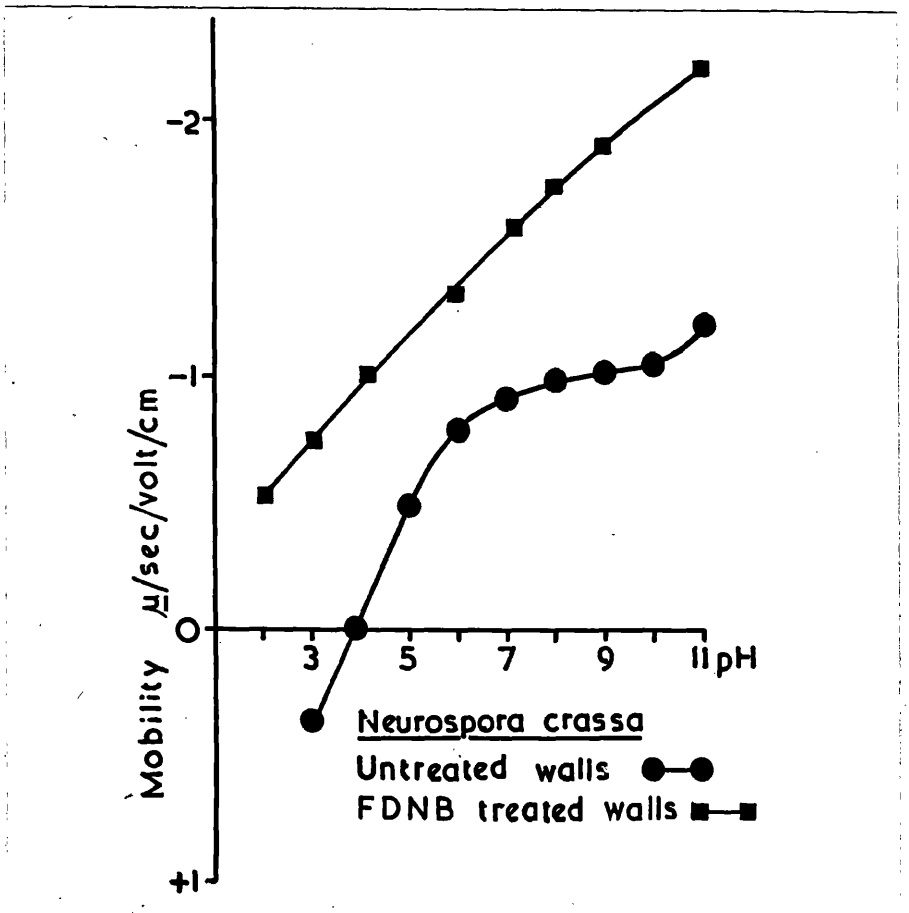


Figure 20. pH/mobility curves of walls of conidia of Neurospora crassa.

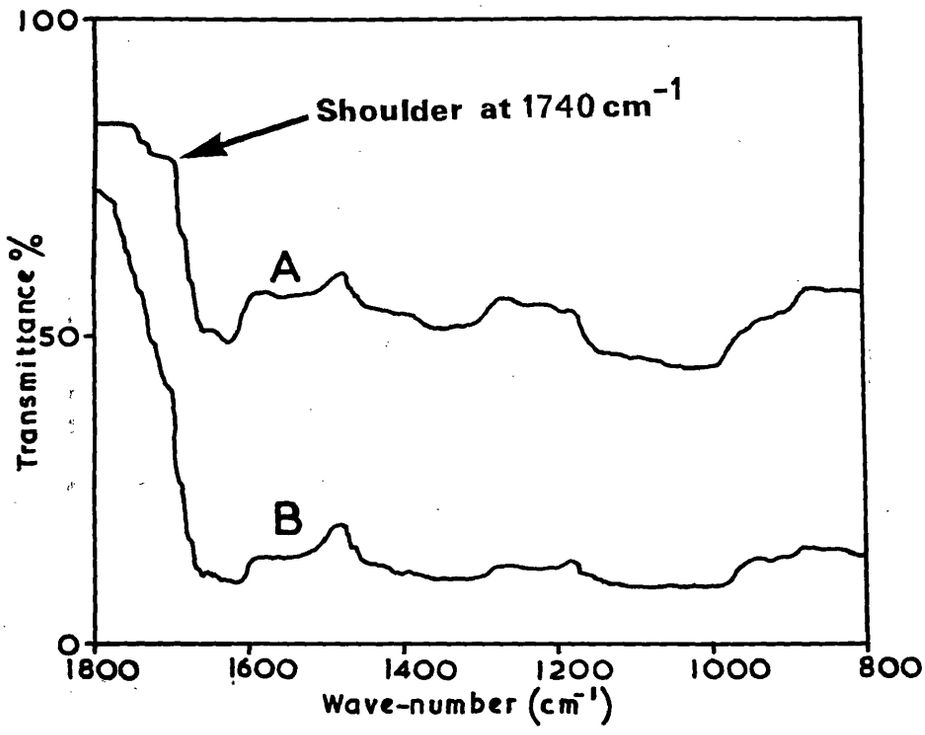


Figure 21. Infrared spectra of Neurospora crassa cell walls. A, methylated with diazomethane: B, walls subjected to the same solvent and drying treatment as A but diazomethane omitted.

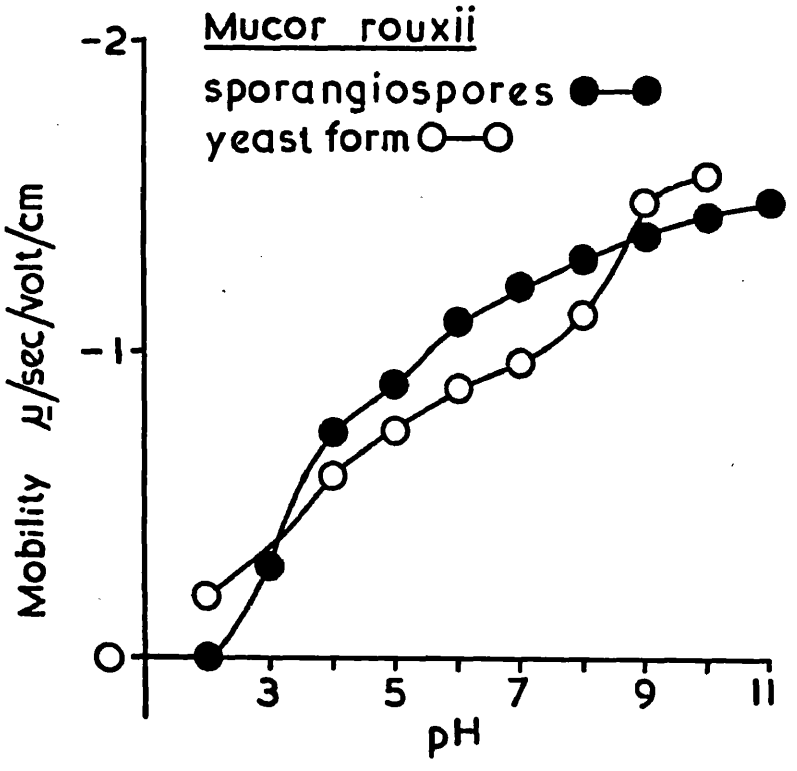


Figure 22. pH/mobility curves of Mucor rouxii.

the yeast-like and filamentous forms (Bartnicki-Garcia and Nickerson, 1962a). The pH/mobility curves for sporangiospores and the yeast-like form are shown in figure 22. Phosphatase treatment had no effect on spore mobility and diazomethane reduced mobility to zero below pH 6 indicating a simple carboxyl surface. The surface of the yeast form was of an extremely acid nature. The presence of a phosphate component was indicated by a reduction in mobility to zero at pH 3.0 after treatment with alkaline phosphatase. Attempts to measure the mobility of the filamentous form were unsuccessful, the particles tending to orientate with the filament pointing away from the positive electrode. The velocity of the particle was approximately inversely proportional to the filament length. The morphology of the three forms of M.rouxii is compared in plates 18,19 and 20.

Plate 18.
Mucor rouxii
Sporangiospores X 1000.

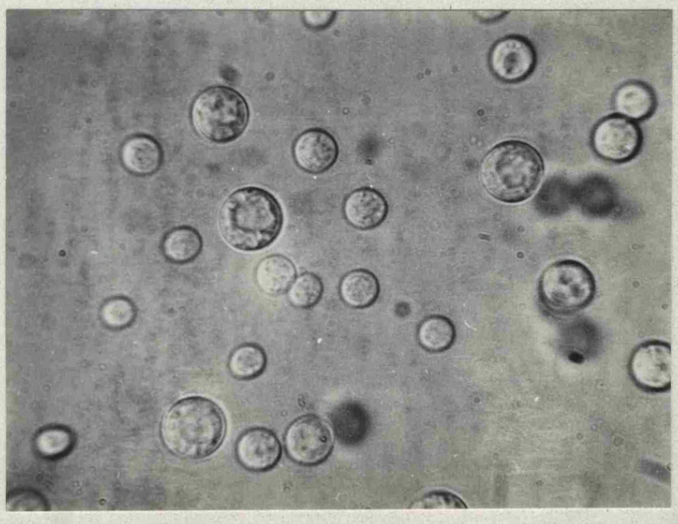


Plate 19.
Mucor rouxii
Yeast-like form X 1000.

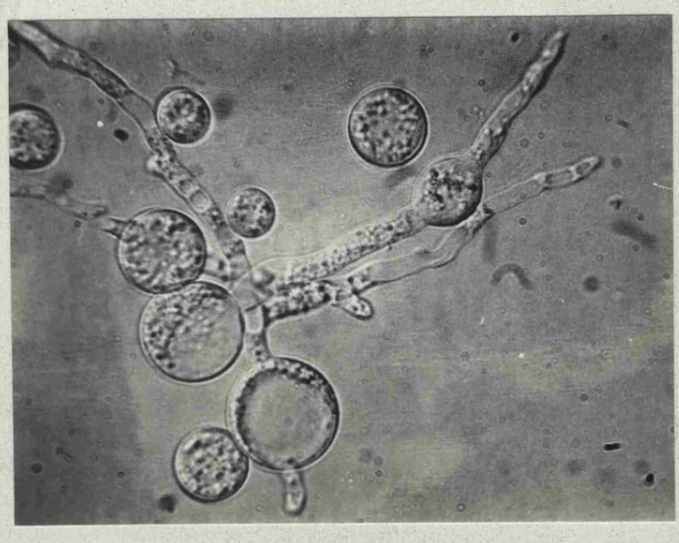


Plate 20.
Mucor rouxii
Partial conversion of yeast-like form to filamentous form X 1000.

Protoplasts were prepared from mycelium of Neurospora crassa and Alternaria tenuis and from conidia of Botrytis fabae. The pH/mobility curves, measured using buffers containing 0.58M sucrose, have isopotential points (3.9 to 4.0) typical of a protein surface (Figure 23). Protoplasts are instantly lysed in the absence of sucrose but mobilities were corrected to correspond to buffer alone. It was assumed that a simple inverse relationship exists between mobility and viscosity (Powney and Wood, 1940) and that other factors such as particle size and charge remain constant. Protoplasts

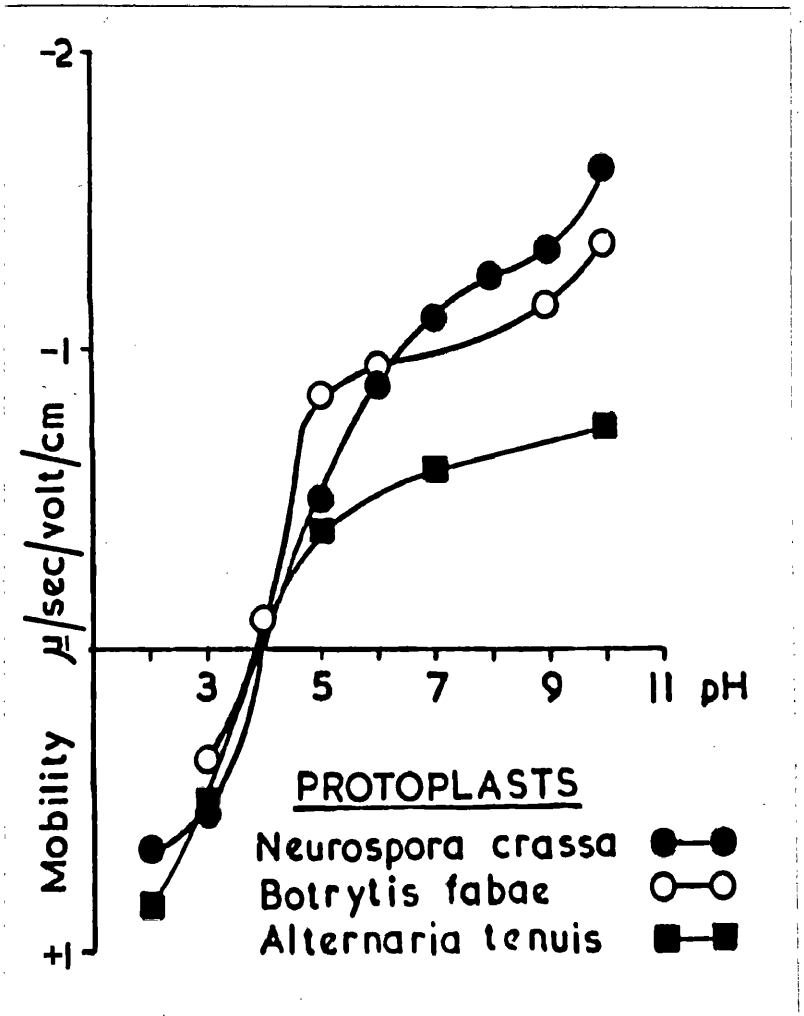


Figure 23. pH/mobility curves of hyphal protoplasts of Neurospora crassa and Alternaria tenuis, and conidial protoplasts of Botrytis fabae.

from B.fabae represent only part of the cell contents (Plate 21), being liberated from the germ tube of germinating conidia by constriction of portions of the protoplasm (Plate 22). Approximately 15 protoplasts are produced from one B.fabae conidium. The absence of cell wall was confirmed by electron microscopy (Plates 23 and 24).

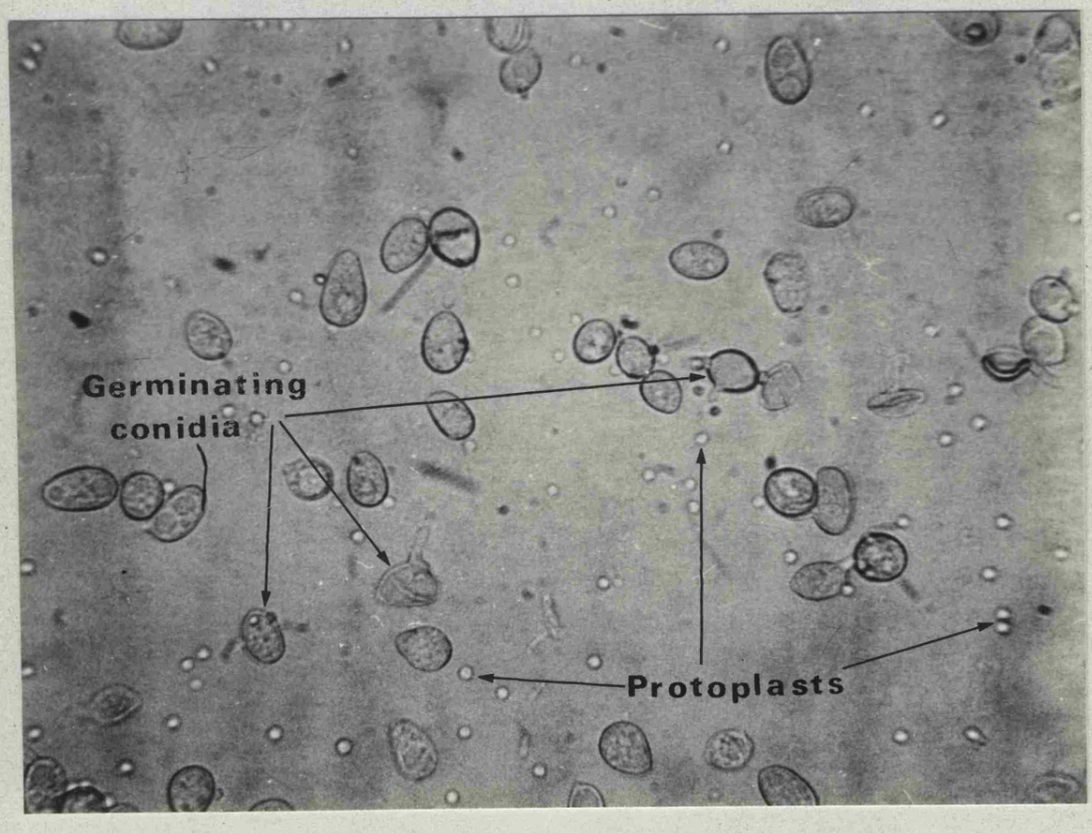


Plate 21. Formation of Botrytis fabae protoplasts from germinating conidia X 600.

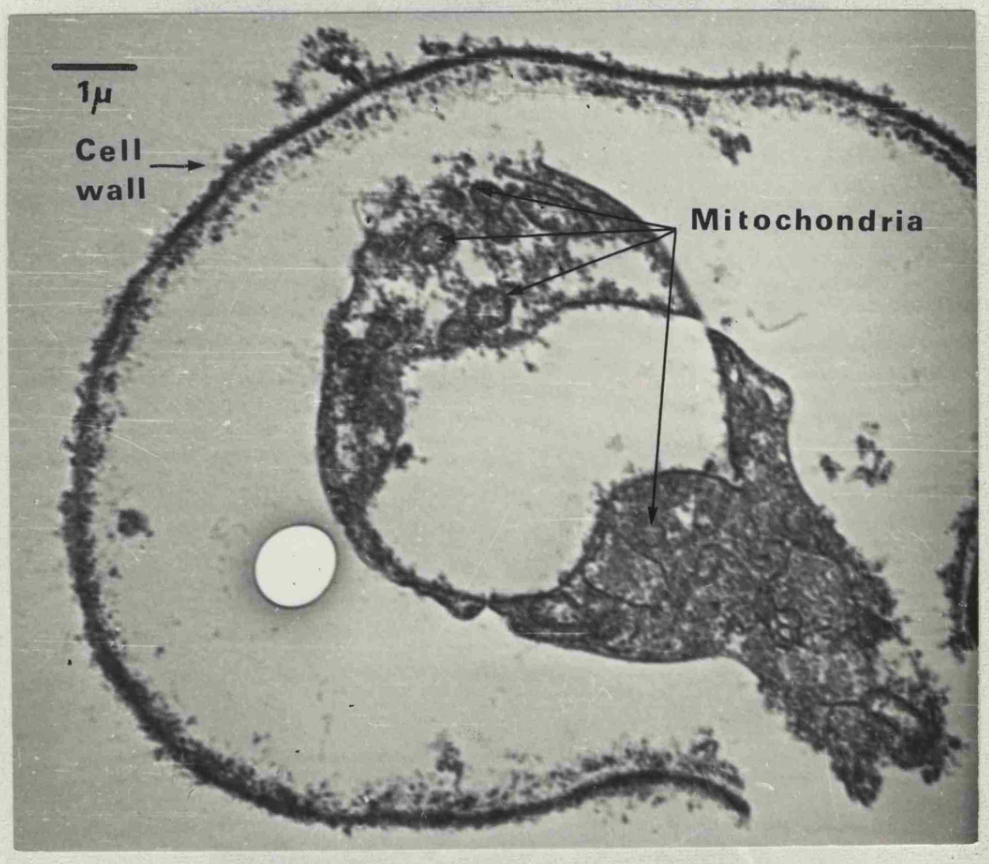


Plate 22. Formation of Botrytis fabae protoplasts. Protoplasm breaking up and flowing through ruptured cell wall.

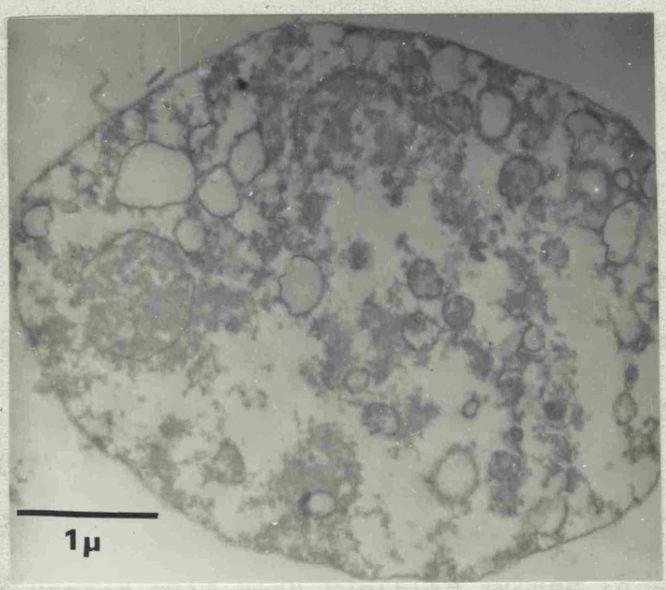


Plate 23. Section through conidial protoplast of Botrytis fabae demonstrating the absence of cell wall.

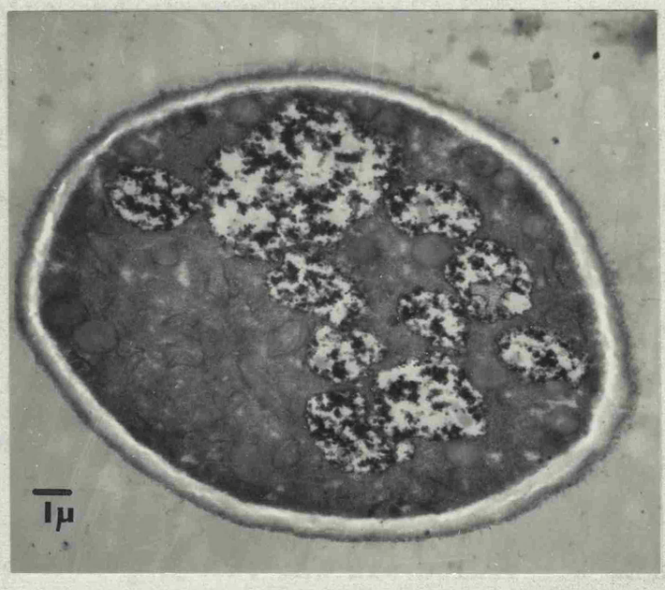


Plate 24. Section through Botrytis fabae conidium showing normal cell wall structure.

Discussion

All the fungal spores examined have characteristic and distinct electrophoretic behaviours. Encysted zoospores of Phytophthora infestans, conidia of Nectria galligena and Verticillium albo-atrum, uredospores of Puccinia lapsanae and teleutospores of Urocystis anemones, showed no positive mobility at low pH suggesting a preponderance of acidic surface groups. Conidia of Botrytis fabae, Podosphaera leucotricha, Erysiphe graminis and Venturia inaequalis, uredospores of Puccinia pruni-spinosae and sporangia of Pseudoperonospora humuli have high positive values in acid solution indicating a mixed amino-carboxyl surface. Chemical treatment of Alternaria tenuis and Botrytis fabae conidia showed phosphate to be absent from the surface and confirmed the presence of amino and carboxyl groups. Amino, carboxyl and phosphate groups have been shown to be integral parts of the surface of Neurospora crassa conidia. The amino groups on the spore surfaces are probably a part of the protein components of the cell wall (Aronson, 1965; Manocha and Colvin, 1967) although aminopolysaccharides may also be present (Harold, 1962a). Carboxyl groups may derive from proteins, polysaccharides (Applegarth, 1967) or uronic acids (Mitchell and Scurfield, 1967). The surface amino groups on Neurospora crassa were derived from ε-lysine and histidine. In addition to these two amino acids leucine was identified on the surface of Botrytis fabae, and leucine and tyrosine on the surface of Alternaria tenuis. Tyrosine is a melanin precursor (Thomson,1962) and therefore the presence of this amino acid in the dark coloured wall of A.tenuis is not surprising.

The high positive mobility of A.tenuis at low pH together with negative mobility after treatment with FDNB suggests that A.tenuis has a higher proportion of surface amino groups than B.fabae. No qualitative differences were found in the surface components of A.tenuis spores of different ages using the electrophoretic technique.

Clean cell walls of Botrytis fabae are electrophoretically similar to intact conidia and apparent differences between Alternaria tenuis cell walls and whole cells have been shown to be due to cytoplasmic contamination. In contrast to intact conidia, purified cell walls of Neurospora crassa have no acid phosphate components, suggesting that the intensive-washing procedure may remove phosphate groups. Eddy and Rudin (1958) prepared yeast cell walls using a less rigorous washing procedure and showed phosphate groups to be present on both intact cells and cell walls. Harold and Miller (1961) found that synthetic polyphosphate could bind to isolated cell walls of N.crassa conidia, at sites later identified as being the amino groups of proteins and galactosamine polymers (Harold 1962a).

Modification of the surface of Stereum purpureum basidiospores with diazomethane and absence of reaction with alkaline phosphatase shows that the only ionised groups present are carboxyl. The surface must be protein-free and probably consists entirely of carbohydrate. The absence of ionogenic groups from sporangia of Phytophthora infestans is consistent with the work of Chapman and Vujičić (1965), who showed that young sporangia of Phytophthora erythroseptica have a

structureless, electron-transparent outer layer consisting probably of cellulose, but constrasts with the amino-carboxyl surface of sporangia of Pseudoperonospora humuli. Confirmation of the differing surface components of spores of closely related species is afforded by the constrasting electrophoretic behaviour of the two rust spores examined.

The sporangiospore wall of Mucor rouxii consists mainly of a neutral glucan while the yeast-like form contains chitosan, a non-acetylated glucosamine polymer, and 12% of uronic acids. The wall of the yeast form also has ten times as much phosphate as the sporangiospore wall (Bartnicki-Garcia and Reyes, 1964, 1968b). The electrophoretic technique shows that both the yeast-like form and the sporangiospores have an acidic surface. This suggests that the amino groups of the chitosan in the yeast-like form may be neutralised by phosphate groups and the carboxyl groups of polyuronides.

The production of a number of small protoplasts from a Botrytis fabae conidium is of interest. Other instances of multiple protoplast production from hyphal and yeast cells are known (Villanueva, 1966). The exact mechanism of protoplast formation from the B.fabae spore is not clear but it has been demonstrated that the weakened spore wall is ruptured during germination and small protoplasts are ejected. The protein-like pH/mobility curves of the fungal protoplasts are in agreement with the lipoprotein constitution of fungal cytoplasmic membranes (Villanueva,1966). The curves are similar to those reported for rat liver nuclei by Vassar et al. (1967) and bacterial protoplasts by James et al. (1965)

and are in conformity with modern theories of membrane structure (Korn, 1966; Robertson, 1969; Staehelin and Probine, 1970; Finean, Bramley and Coleman, 1971).

P A R T II

THE SURFACE LIPIDS OF SOME FUNGAL SPORES

PART II

THE SURFACE LIPIDS OF SOME FUNGAL SPORES

The presence of ionogenic groups on the surface of fungal spores was demonstrated in Part I; lipids may also be present. Although the lipid composition of the mycelium of many fungi has been studied in detail (Shaw, 1966) little is known of the lipid components of fungal spores. Palmitic, stearic, oleic and linoleic acids are the major fatty acids of uredospores of various rust species (Tulloch, Craig and Ledingham, 1959; Tulloch and Ledingham, 1962, 1964a, 1964b). These acids are also present in conidia of Fusarium species (Rambo and Bean, 1969) and sporangiospores of Mucor and Rhizopus species (Sumner and Morgan, 1969; Weete, Weber and Laseter, 1970). Weete, Laseter, Weber, Hess and Stocks (1969) determined the relative proportions of hydrocarbons in chlamydospores of four different rust fungi and showed the C_{27}, C_{29} and C_{31} alkanes to predominate.

Few attempts have been made to distinguish cell wall lipid from intracellular fractions or to determine the distribution of the lipid within the wall. Bertaud *et al.* (1963) analysed benzene washings of *Pithomyces chartarum* spores. This fraction differed in composition from the total spore lipid and they concluded that surface material had been removed. Oró *et al.* (1966) reported the composition of surface hydrocarbons from smut spores. Laseter *et al.* (1968a) examined the fatty acids and alkanes from three different Tilletia species but were unable to detect any differences in external morphology between normal spores and spores free of surface lipid using a freeze-etching technique. The study of fine

structure can indicate the location of cellular lipids: for example McKeen, Mitchell and Smith (1967) found lipid bodies adjacant to the conidal wall of Erysiphe cichoracearum.

The existence of surface lipid on biological particles can be detected by the increase in mobility produced in the presence of anionic surface-active agents (James, 1965b). This electrophoretic technique has been used in the present studies to investigate the presence of surface lipid on a range of fungal spores and isolated spore walls. Spores of the species shown by electrophoresis to possess surface lipid were further examined. A comparison was made of the fatty acid and alkane components of a surface extract and of the corresponding wall fractions.

Materials and Methods

Fungal material

Alternaria tenuis Nees, Botrytis fabae Sardiña, Nectria galligena Bres., Neurospora crassa Shear and Dodge macroconidial wild type Em 5297a, Mucor rouxii (Calmette) Wehm. and Verticillium albo-atrum Reinke and Berth. were cultured on the medium indicated in Part 1. Rhizopus stolonifer (Ehrenb. ex Fr.) Lind and Penicillium expansum Link ex Thom were grown on 2% malt agar. Spores were harvested after seven days growth by the method previously described (page 25). Conidia of Erysiphe graminis DC. ex Mérat were washed from naturally infected oat seedlings (var. Black Supreme) and conidia of Erysiphe cichoracearum DC. from infected marrow plants (var.Green bush). The culture of R.stolonifer was obtained from the Botany Department, University of Bristol. P.expansum was from the culture collection of Bath University and E.cichoracearum was

supplied by Dr. E.C. Hislop (Long Ashton Research Station). The origins of the other species are indicated in Part I. Spores which had been shown to possess surface lipids by the electrophoretic technique were harvested from cultures on 500 - 1500 petri dishes and freeze dried.

Microelectrophoresis

Electrophoretic mobilities were measured using the apparatus previously described (page 25). Surface lipids were detected by studying the variation in the mobility of particles with the concentration of sodium dodecyl sulphate (SDS) in the suspension medium (Hill et al., 1963b). Cells and cell walls were washed once in phosphate buffer (pH 7.0,I:0.01) before suspension in this buffer or in buffers containing 10^{-6}M, 10^{-5}M and 10^{-4}M sodium dodecyl sulphate. These concentrations of SDS were not high enough to significantly after the pH, ionic strength or conductivity of the buffer solution.

Extraction procedures

Surface lipids were removed by suspending the freeze dried spores in light petroleum (boiling range 40 - 60°) at room temperature for one minute. The supernatant fraction after centrifugation was decanted and filtered through closely packed ether-extracted cotton wool to remove any residual spores. Purified cell walls were prepared from the treated spores using the Mickle disintegrator (page 25), and dried over phosphorous pentoxide. Wall lipids were subsequently removed by extraction with chloroform: methanol (2:1, v/v) for 18 hours in a Bolton extractor.

Thin-layer chromatography

Preliminary investigations were carried out on Silica Gel G (Merk) using light petroleum: diethyl ether: acetic acid

(90:10:1, by vol.) (Malins and Mangold, 1960). Standards representative of free fatty acids (stearic acid), phospholipids (lecithin), hydrocarbons (n-tetracosane), sterols (cholestrol), steryl esters (cholestrol palmitate) and triglycerides (tristearin) were included. Compounds were detected by spraying the plate with chlorosulphonic acid: glacial acetic acid (1:2, v/v) (Krebs, Heusser and Wimmer,1969) and charring in an oven at 200°. Preparative scale separations into fatty acid and hydrocarbon fractions were carried out on silica gel using the same solvent system. Fatty acids were methylated by the addition of freshly prepared diazomethane in ether before gas-liquid chromatography. The solvent and excess reagent were removed by evaporation.

Gas-liquid chromatography

A Hewlett-Packard 5750 gas chromatogram fitted with dual columns and flame ionisation detectors and 0-1 mV recorder were used. Retention data were determined using both OV210 (3% loading) and E301 (5% or 8% loading) on 80-100 mesh Chromosorb W-HP. The chromatographic columns were of stainless steel 2 mm internal diameter and 1.8 m in length. The carrier gas was nitrogen, flow rate 40 ml/minute (70 p.s.i.) Analyses were made using temperature programming over the ranges indicated in the tables. Relative retention times of the peaks were determined using n-tricosane as a standard. Relative peak areas were measured from the chart by the triangulation method.

Results

Electrokinetic measurements

Surface lipid was detected on only four of the ten species examined (Table 1). The electrophoretic mobility of spores of Alternaria tenuis, Botrytis fabae, Neurospora crassa and

Table 1. The effect of sodium dodecyl sulphate (SDS) on the electrophoretic mobility of spores and isolated spore walls.

	Electrophoretic mobility μ/sec/volt/cm				% Increase at 10^{-4} M SDS
SDS conc.	0	10^{-6}M	10^{-5}M	10^{-4}M	
Intact spores					
Alternaria tenuis	2.62	3.02	3.04	3.26	24.4
Botrytis fabae	1.54	1.62	1.65	1.80	16.9
Erysiphe cichoracearum	.80	.82	.76	.76	Nil
Erysiphe graminis	3.54	-	3.56	3.60	1.6
Mucor rouxii	1.72	1.83	1.83	1.79	3.9
Nectria galligena	3.75	-	3.70	3.70	Nil
Neurospora crassa	.45	.48	.49	.56	24.4
Penicillium expansum	1.54	1.80	1.76	1.76	14.3
Rhizopus stolonifer	1.78	1.83	1.86	2.37	33.1
Verticillium albo-atrum	1.27	1.28	1.29	1.26	Nil
Spore walls.					
Alternaria tenuis	1.55	1.58	1.61	3.29	112.2
Botrytis fabae	1.41	1.45	1.49	1.53	8.5
Neurospora crassa	.69	-	1.23	1.59	130.4
Penicillium expansum	1.52	-	1.57	1.56	2.6

- = not determined

Rhizopus stolonifer rose progressively in increasing concentrations of SDS, showing the presence of some surface lipid. The mobility of Penicillium expansum conidia in 10^{-6}M SDS also increased but the absence of any further rise at higher SDS levels indicated the probable absence of surface lipid. The mobility of spores of the other species were constant within the limits of experimental error. Mobilities of isolated cell walls confirmed results obtained with whole spores.

Surface lipids

Surface lipids from the freeze dried spores of Alternaria tenuis, Botrytis fabae, Neurospora crassa and Rhizopus stolonifer varied between 0.15% and 0.2% of the spores dry weight (Table 2). No surface lipid was found on Penicillium expansum conidia, confirming the electrophoretic results. The bulk of the surface extract was shown to consist of free fatty acids by thin-layer chromatography. Phospholipids and hydrocarbons were also present.

Table 2. The surface lipid composition of some fungal spores.

	Surface extract as % spore dry weight.
Alternaria tenuis	0.15
Botrytis fabae	0.19
Neurospora crassa	0.17
Penicillium expansum	Not detected
Rhizopus stolonifer	0.20

Analysis of fatty acid fractions by gas-liquid chromatography showed a preponderance of saturated acids with even numbers of carbon atoms. Palmitic and stearic acids predominated (Table 3). Analysis of hydrocarbon fractions showed approximately equal amounts of the "odd" and "even" series of carbon numbers to be present on the surface of the spores of each of the four species

Table 3. The relative composition of the surface fatty acids of fungal spores (methylated and chromatographed on 5% E301 programmed 150-250°).

T_{rel} tricosane	A. tenuis	B. fabae	N. crassa	R. stolonifer	Identity
0.13	0.40	-	-	-	C_{12} 1Δ
0.17	-	-	-	trace	
0.19	2.17	3.09	3.12	3.12	C_{12}
0.25	0.32	0.80	0.78	trace	C_{13} 1Δ
0.28	trace	0.36	0.49	trace	C_{13}
0.35	1.36	3.35	trace	1.56	C_{14} 1Δ
0.37	8.43	8.76	10.33	8.53	C_{14}
0.42	0.44	0.81	1.07	trace	
0.45	1.28	1.21	1.23	1.04	C_{15} 1Δ
0.48	1.69	1.47	2.34	1.77	C_{15}
0.50	3.21	5.23	1.15	3.85	
0.56	2.49	5.50	-	3.64	C_{16} 1Δ
0.58	28.73	27.95	40.26	41.25	C_{16}
0.63	1.48	1.79	3.20	2.08	
0.66	1.81	2.59	2.67	2.45	C_{17} 1Δ
0.70	2.41	1.34	1.61	2.71	C_{17}
0.78	3.01	2.10	trace	2.71	C_{18} 1Δ
0.80	14.81	11.18	17.43	14.88	C_{18}
0.89	2.56	1.61	1.39	2.08	C_{19}
0.97	1.48	3.13	2.22	-	C_{20} 1Δ
1.01	5.45	-	1.48	2.18	C_{20}
1.05	1.52	-	-	2.71	C_{21} 1Δ
1.12	1.00	2.19	1.27	trace	C_{21}
1.14	0.88	-	-	-	
1.20	0.96	2.73	0.66	-	C_{22} 1Δ
1.24	6.17	1.07	2.79	3.43	C_{22}
1.32	1.76	2.10	1.64	trace	C_{23}
1.39	-	-	1.64	-	C_{24} 1Δ
1.42	2.00	1.43	1.23	trace	C_{24}
1.47	trace	1.16	-	-	C_{25} 1Δ
1.50	1.28	3.40	-	-	C_{25}
1.56	0.84	1.57	-	-	C_{26} 1Δ
1.59	trace	2.06	-	-	C_{26}

1Δ = mono unsaturated, - = not detected

examined in detail (Table 4). The distribution patterns varied considerably. Surface alkanes from A.tenuis showed a random distribution with major peaks corresponding to C_{19}, C_{20}, C_{21}, C_{23}, C_{25}, C_{26}, and C_{28}. Alkanes from the surface of B.fabae and N.crassa showed concentration maxima at the lower end of this series with main peaks at C_{20}, C_{21} and C_{22}. R.stolonifer showed a maximum distribution in the C_{21} - C_{25} range. Virtually no branched chain or unsaturated compounds were detected.

Wall lipids

The amounts of lipid extracted from purified cell walls and approximate values for the total fatty acid components are given in Table 5. The spore wall of Penicillium expansum has a comparatively small amount of lipid (1.1%). Other Penicillium species have similar lipid contents. Van Etten and Gottlieb (1965) found 1.6% total lipid in Penicillium atrovenetum spores, and Applegarth (1967) 1-2% lipid in Penicillium notatum hyphal walls. In the present investigation thin-layer chromatography showed fatty acids, phospholipids and hydrocarbons to be present in wall extracts. No detailed analysis of phospholipid fractions was carried out.

Analysis of the fatty acid fractions from spore walls of Alternaris tenuis, Botrytis fabae, Neurospora crassa and Rhizopus stolonifer by gas-liquid chromatography (Table 6) showed that their relative compositions differed from those of the corresponding surface fractions. Palmitic and stearic acids again predominated. The fatty acid composition of Penicillium expansum walls differed considerably from that of the other spores investigated. A high degree of unsaturation of the fatty acids was evident, amounting to 45.7% of those identified.

Table 4. The relative composition of the surface hydrocarbons of fungal spores (chromatographed on 8% E301 programmed 180 - 306°).

T_{rel} tricosane	A. tenuis	B. fabae	N. crassa	R. stolonifer	Identity*
0.42	3.21	1.13	-	trace	C_{18}
0.53	11.24	2.27	8.11	0.21	C_{19}
0.60	-	-	1.87	-	
0.65	14.46	13.63	28.06	5.19	C_{20}
0.77	12.05	22.73	21.04	12.44	C_{21}
0.88	4.82	19.31	10.91	15.56	C_{22}
0.96	2.41	-	-	-	
1.00	8.03	11.36	6.24	14.52	C_{23}
1.12	6.43	10.22	4.99	15.56	C_{24}
1.24	10.04	5.68	4.36	13.06	C_{25}
1.36	8.03	4.54	3.11	8.30	C_{26}
1.48	1.61	2.27	3.11	6.23	C_{27}
1.58	8.03	6.81	3.89	4.15	C_{28}
1.68	4.82	trace	1.87	2.60	C_{29}
1.77	2.41	-	1.25	1.25	C_{30}
1.87	2.41	-	0.70	0.62	C_{31}
1.96	-	-	0.47	0.31	C_{32}
Carbon preference index	.99	1.05	.80	.86	

- = not detected

*all hydrocarbons definitely identified were n-alkanes

Table 5. The lipid composition of some fungal spore walls.

	Wall lipid as % wall dry weight.	Fatty acids as % wall lipid
Alternaria tenuis	1.67	18
Botrytis fabae	5.08	31
Neurospora crassa	8.95	6
Penicillium expansum	1.10	31
Rhizopus stolonifer	3.97	12

Table 6. The relative composition of the wall fatty acids of fungal spores (methylated and chromatographed on 3% OV210 programmed 100 - 230°).

T_{rel} tricosane	A.tenuis	B.fabae	N.crassa	P.expansum	R.stolonifer	Identity
0.36	2.78	4.69	3.20	1.36	5.33	C_{12}
0.52	-	0.39	1.28	2.72	-	C_{14} 1Δ
0.57	4.86	6.26	4.27	1.96	6.00	C_{14}
0.63	1.39	0.39	0.85	-	-	
0.66	4.63	3.91	2.35	2.26	3.00	C_{15}
0.70	5.32	1.17	1.78	trace	-	C_{16} 1Δ
0.80	41.66	22.69	47.29	30.57	33.33	C_{16}
0.83	-	3.26	2.84	0.83	3.00	
0.89	4.86	5.61	6.47	1.74	-	C_{17}
0.91	7.41	-	trace	1.66	-	
0.98	4.86	9.64	4.55	38.72	12.00	C_{18} 1Δ
1.02	22.22	10.82	13.51	11.47	18.33	C_{18}
1.06	-	trace	-	0.98	6.00	C_{19} 1Δ
1.09	-	trace	-	-	8.00	C_{19}
1.18	-	-	4.27	-	-	C_{20} 1Δ
1.22	-	1.43	2.49	-	1.66	C_{20}
1.33	-	1.30	1.99	-	-	C_{21}
1.42	-	-	1.42	2.11	1.33	C_{22} 1Δ
1.44	-	-	1.42	2.49	2.00	C_{22}
1.49	-	8.60	-	-	-	C_{23} 1Δ
1.52	-	16.30	-	-	-	C_{23}
1.61	-	3.52	-	1.13	-	

1Δ = mono unsaturated, - = not detected

The principal acid was oleic, though considerable amounts of palmitic and stearic acids were also present.

Although it was not possible to make an accurate quantitative assessment of the hydrocarbon content of the walls it was evident that only extremely small amounts were present. The pattern of distribution of the identified normal alkanes of the whole wall (Table 7) differed widely from that of the surface fractions (Figure 24). The large proportion of the hydrocarbons not definitely identified indicated a high level of branched chain or unsaturated components.

Discussion

The microelectrophoretic technique used by other workers to detect surface lipids on a variety of biological particles, has been extended to include a range of fungal spores. The extremely easy removal of lipids from the surface of Alternaria tenuis, Botrytis fabae, Neurospora crassa and Rhizopus stolonifer spores by solvent washing confirmed the electrophoretic results. Surface lipids, where present, represent only a small proportion of the total wall lipids.

Various extraction procedures have been used to remove surface lipid from fungal spores. Bertaud et al. (1963) extracted fatty acids from the surface of Pithomyces chartarum spores by ten successive benzene washes each of one minute duration, while Laseter et al. (1968b) have reported the removal of surface material from spores of the smut Ustilago maydis with benzene:methanol (3:1 v/v) for 30 minutes at 50°. Palmitic, oleic and linoleic acids were the main fatty acid components in both instances but the possibility that material not originating from the surface may have been

Table 7. The relative composition of the wall hydrocarbons of fungal spores (chromatographed on 3% OV210 programmed 100 - 230°).

T_{rel} tricosane	A. tenuis	B. fabae	N. crassa	P. expansum	R. stolonifer	* Identity
0.23	1.02	0.56	-	trace	0.55	C_{16}
0.28	-	1.26	-	trace	0.55	
0.32	2.05	2.10	3.15	-	3.28	C_{17}
0.34	1.54	0.84	0.70	3.57	1.64	
0.39	trace	-	0.70	-	-	
0.42	2.04	1.20	3.15	3.34	5.46	C_{18}
0.46	1.02	2.10	2.79	6.69	3.28	
0.53	3.07	1.68	2.10	2.68	2.45	C_{19}
0.57	4.61	2.93	2.79	1.79	1.09	
0.62	-	0.28	-	-	-	
0.64	2.04	0.84	2.10	2.01	1.63	C_{20}
0.68	6.14	6.71	10.61	8.92	5.73	
0.76	4.61	2.10	2.10	1.79	1.37	C_{21}
0.83	-	16.36	-	-	-	
0.86	13.83	12.86	0.47	9.37	7.65	
0.88	2.56	1.96	11.19	8.04	2.46	C_{22}
0.92	26.84	-	2.44	21.43	13.11	
1.00	4.61	3.36	4.20	trace	4.91	C_{23}
1.05	1.02	5.46	0.93	2.68	trace	
1.10	14.34	7.32	9.32	8.92	6.55	C_{24}
1.21	2.25	2.52	3.50	2.68	4.92	C_{25}
1.32	3.79	4.20	5.83	2.68	6.83	C_{26}
1.41	1.54	3.92	5.59	2.68	4.92	C_{27}
1.51	-	2.10	6.88	-	-	
1.53	1.02	3.36	0.70	-	2.73	C_{28}
1.55	-	0.56	-	-	-	
1.61	-	4.90	7.46	2.68	12.29	C_{29}
1.67	-	2.23	2.91	-	-	
1.69	-	2.79	1.75	4.69	3.28	C_{30}
1.78	-	2.10	5.24	1.34	3.28	C_{31}
1.88	-	0.84	-	trace	-	
1.90	-	0.56	1.40	2.00	-	C_{32}
Carbon preference index	1.26	0.72	1.00	0.94	0.47	

- = not detected

* all hydrocarbons definitely identified were n-alkanes

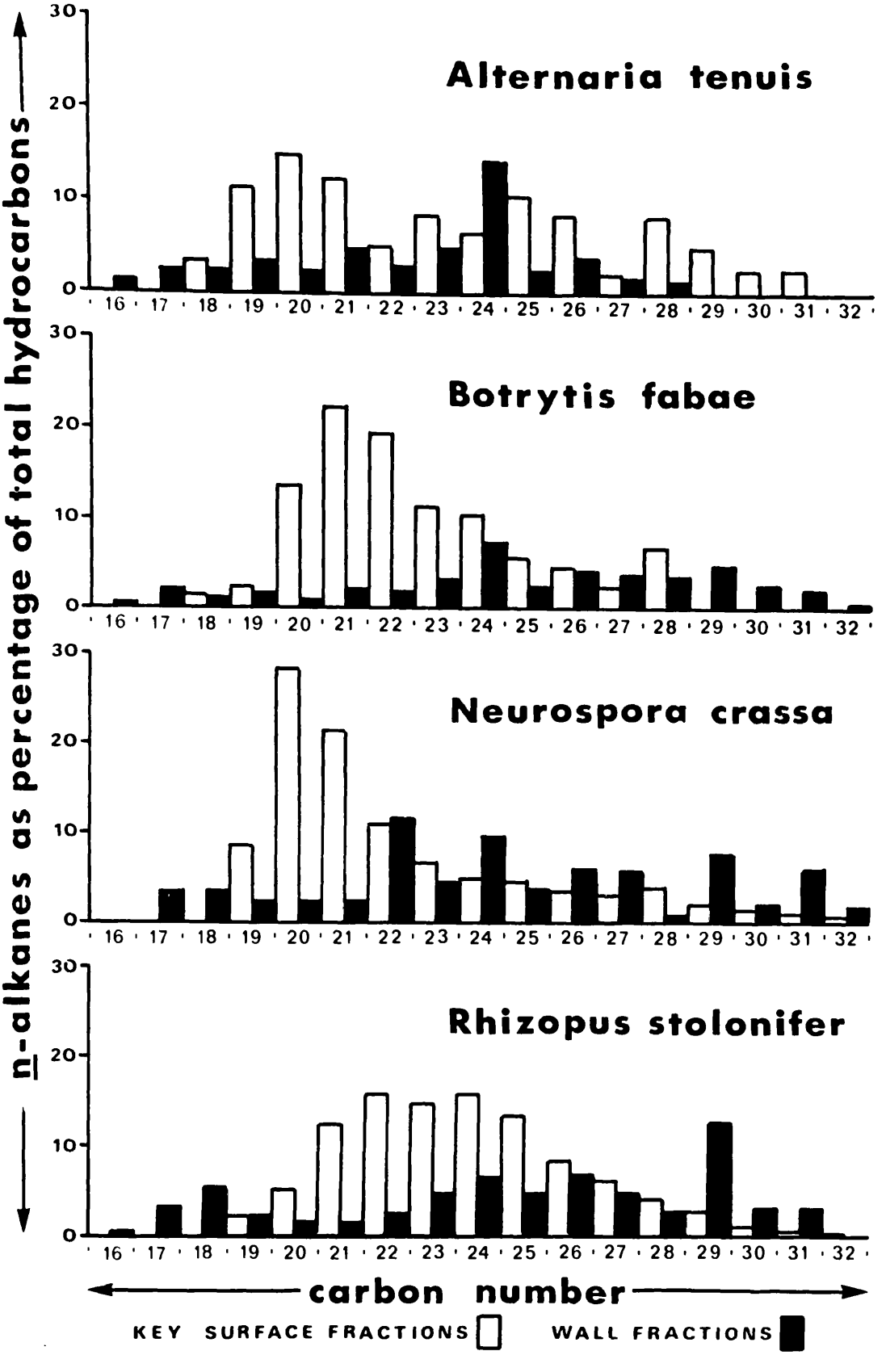


Figure 24. Comparison of surface and wall alkanes of some fungal spores.

extracted cannot be ignored. In the present investigation surface material was removed by a single wash with light petroleum for one minute at room temperature. This less rigorous procedure is similar to that employed by Silva Fernandes, Baker and Martin (1964) in studies of surface waxes on leaf material. The failure of the technique to remove material from Penicillium expansum spores, confirmed that only surface fractions were extracted.

Palmitic and stearic acids were major fatty acid components of the surface material removed from all the spores examined. The main surface fatty acid components were similar to those of the wall lipid fraction although the presence of the C_{23} and $C_{23}1\Delta$ acids in Botrytis fabae walls was unexpected. No trace of 9,10-epoxyoctadecanoic acid, shown by Jackson and Frear (1966) to be a major component of the fatty acids in rust uredospores, was found in any of the spore walls. Even carbon-number straight chain acids predominated in all the fractions analysed. A high level of unsaturation in the wall fatty acids of Penicillium expansum is typical of the genera. Fatty acids from spores of Penicillium atrovenetum (Van Etten and Gottlieb, 1965) and mycelium of Penicillium pulvillorum (Nakajima and Tanenbaum, 1968) and Penicillium cyanum (Koman Betina and Baráth, 1969) are similar.

The alkanes present in spore surface washings ranged from C_{18} to C_{32} in chain length. The major peaks of the surface and corresponding wall fractions do not coincide (Figure 24). Carbon preference ratios were of the order of unity in surface extracts. In contrast with the surface fractions a large proportion of the wall hydrocarbons were not definitely identified, indicating a high degree of unsaturation

or branching. Carbon preference indices of alkanes in wall fractions consequently have little meaning. Oró et al. (1966) reported that in alkanes removed from the surface of Ustilago maydis, Ustilago nuda and Sphacelotheca reiliana chlamydospores odd carbon chain lengths predominated. Detailed differences were evident but the major peaks occurred at C_{25}, C_{27}, C_{29} and C_{31}. Laseter et al. (1968b) found considerable differences in the relative proportions of surface alkanes from spores of U.maydis obtained from different sources, though the major peaks were again reported at C_{25}, C_{27}, C_{29} and C_{31} in spores of three Tilletia species by Laseter et al. (1968a). An almost identical pattern was obtained from the uninfected host. These workers suggest that Tilletia remove hydrocarbons directly from the host and incorporate them unchanged into the spores, but an alternative possibility is that the alkanes may have been removed from the leaf surface when the spores were gathered.

An equal distribution of odd and even series alkanes in the total lipids of Penicillium species, Aspergillus species, Trichoderma viride and Saccharomyces species was found by Jones (1969). Merdinger and Devine (1965) showed a similar situation in the lipids of the yeast Debaryomyces hansenii. Stránský, Streibl and Herout (1966) concluded that, unlike higher plants, those at a low level of evolution have a ratio of odd to even numbered alkanes approximating to one. Herbin and Robins (1969) showed however, that in higher plants when n-alkanes form only a small percentage of the leaf cuticular wax, the dominance of odd over even carbon number chain lengths tends to disappear.

The differences which Stránský *et al.* (1966) ascribed to plants of higher and lower evolutionary levels could be more a reflection of the variation between internal and external lipids. The present work provides evidence of distinct surface lipids on spores of *Alternaria tenuis*, *Botrytis fabae*, *Neurospora crassa* and *Rhizopus stolonifer*, and supports the view that lower plants do not show an odd alkane preference.

All the spores shown to possess surface lipids are air borne and difficult to wet. The absence of surface lipids from spores which are water dispersed such as *Verticillium albo-atrum* and *Nectria galligena* is not surprising, but others from which surface lipids are absent are nevertheless hydrophobic. Douglas *et al.* (1970) working with actinomycete spores have confirmed that water-repellent properties are not necessarily associated with surface lipids. The water-repellent properties of Penicillium conidia have been attributed to the presence of ether-soluble cyclic peptides on the surface (Bertaud *et al.*, 1963). The absence of an extractable surface fraction and the small amount of material soluble in chloroform and methanol mixture in the cell walls suggests that the water-repellent properties are not due to lipids or cyclic peptides. Powdery mildew conidia are also water-repellent and differ from spores of other fungi in their resistance to desiccation (Yarwood, 1936) while spore germination is usually impaired by wetting (Zaracovitis, 1964). McKeen *et al.* (1967), in a study of the *Erysiphe cichoracearum* conidium, considered that the impervious outer layer of the spore played an important role in the action of water and fungicides on mildew conidia. The

pH/mobility curves of Podosphaera leucotricha and Erysiphe graminis (Part I) do not account for the characteristic physical properties of powdery mildew conidia: unionised compounds or the physical conformation of the surface may be responsible for the non-wettable nature of these spores.

P A R T III

THE ELECTROPHORETIC PROPERTIES AND SOME SURFACE COMPONENTS OF PENICILLIUM CONIDIA

PART III

THE ELECTROPHORETIC PROPERTIES AND SOME SURFACE COMPONENTS OF PENICILLIUM CONIDIA

A general survey of the surface ionogenic and lipid components of fungal spores has been made in Parts I and II. A more detailed investigation of the surface components of conidia of Penicillium species is now described in an attempt to correlate electrophoretic and chemical information with taxonomic features.

The chemical composition of hyphal cell walls has been studied in some detail (Bartnicki-Garcia, 1968) but less attention has been paid to the physical and chemical properties of the spore wall. The composition of sporangiospore walls of Mucor rouxii (Bartnicki-Garcia and Reyes, 1964) and conidial walls of Aspergillus oryzae (Horikoshi and Iida, 1964) has been investigated, while Rizza and Kornfeld (1969) have compared the carbohydrate and amino acid composition of conidial and hyphal walls of Penicillium chrysogenum.

The surface ornamentation of fungal spores may be examined by the replica technique (Bigelow and Rowley, 1968), by the use of the scanning electron microscope (Hawker et al., 1970; Campbell, 1969) and by freeze-etching (Laseter, et al., 1968a; Hess and Stocks, 1969). The freeze-etching technique has shown the surface of Penicillium conidia to be covered with a distinctive pattern of rodlets (Hess et al., 1968).

One of the purposes of this investigation is to determine whether closely-related species of Penicillium can be differentiated by their pH/mobility curves. A study is made of

the spores of five Penicillium species using the electrophoretic technique and the effect of virus infection on the surface of Penicillium stoloniferum conidia is investigated. A detailed study of Penicillium expansum spores is made using micro-electrophoresis, chemical analysis, the scanning electron microscope and freeze-etching.

Materials and Methods

Fungal material

Conidia from 7-day cultures of Penicillium expansum Link ex Thom, Penicillium digitatum Sacc., Penicillium roqueforti Thom, Penicillium thomii Maire, three strains of Penicillium notatum Westling and two strains of Penicillium stoloniferum Thom (virus-containing strain ATCC 14586 and the corresponding virus-free isolate ATCC 14586 B3/2), were grown on malt agar and harvested as described in Part I. All fungi originated from the Bath University culture collection with the exception of two strains of P.notatum (Commonwealth Mycological Institute 15378 and 17969) and the two strains of P.stoloniferum from the Biochemistry Department, Imperial College of Science and Technology, London. Young conidia of P.expansum were obtained by removing spores under aseptic conditions from 7-day cultures grown on malt agar, and incubating the washed mycelium at 25° for a further one or two days before the freshly produced spores were harvested. The fungus was examined microscopically after the initial wash to ensure the complete absence of spores. Conidia were also harvested from 7-day cultures of P.expansum grown on Fries medium of composition:agar, 20 g; sucrose, 20 g; $NaNO_3$, 3 g; Na tartrate, 1 g; $MgSO_4.7H_2O$, 0.5 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g; Zn as $ZnSO_4.7H_2O$, 2000 µg; Fe as $FeCl_3.6H_2O$, 200 µg;

Cu as $CuSO_4.5H_2O$, 80 µg; Mn as $MnSO_4.4H_2O$, 20 µg; Mo as $Na_2MoO_4.2H_2O$, 20 µg; biotin 5 µg; distilled water to 1 litre (Richmond and Somers, 1962), containing 0.3, 3.0 and 6.0 g KH_2PO_4 per litre. The pH of the medium was adjusted to 6.0. Cell walls were prepared and washed as previously described (Part I). The final washings were free of ultraviolet absorbing material.

Microelectrophoresis

Electrophoretic mobilities were measured using the apparatus and method previously described (Part I). All spores were washed in the appropriate buffer before electrophoretic measurements. Each mobility was the mean of at least 20 observations: the standard error of the mean was less than 4%.

Treatments to modify the surface groups

Alkaline Phosphatase (EC 3.1.3.1.), diazomethane (DAM) and 1-fluoro-2,4-dinitrobenzene (FDNB) were used to modify conidia as described on pages 31 and 32.

p-Toluenesulphonyl chloride (PTSC). Washed conidia were suspended in barbiturate buffer (pH 7.0; I:0.05) and shaken with 50 mg PTSC for 24 hours at room temperature (Gittens and James, 1963). The suspensions were then decanted from the excess PTSC, the cells washed in the appropriate buffer and the mobility determined.

Extraction of water-soluble wall components

Soluble surface material was removed from spores by repeated washing with water or by using the technique of Dyke (1964) described previously (page 25). Polyphosphates and sugars were examined in the combined water washings after concentration to a volume of 5 ml in a rotary evaporator at 40°.

Analytical methods

Phosphorus: After digestion of organic material with nitric followed by perchloric acid, phosphorus was determined by the formation of the phosphovanado-molybdate complex (Hanson, 1950). The yellow colouration was measured at 470 mμ using a Unicam S.P.600 spectrophotometer.

Polyphosphates:

Polyphosphates were identified by thin-layer chromatography on starch using both rectangular (200 x 200 mm) and circular (250 mm diameter) plates as described by Canic, Turčic, Petrovic and Petrovic, 1965). The solvent was trichloroacetic acid: isopropanol: distilled water containing 2.5mM ethylenediaminetetra-acetic acid and 2.5mM ammonium hydroxide (5:80:40 w/v/v). Polyphosphates were detected by spraying the plate with the molybdate-perchloric acid reagent of Hanes and Isherwood (1949). After exposure to ultraviolet light for 3 minutes polyphosphates were indicated by a blue colouration against the white background. An alternative chromatographic procedure on paper (Miyachi, 1961), using trichloroacetic acid, isopropanol, and water containing 35mM ammonium hydroxide (4:70:30 w/v/v) was also employed. After development papers were sprayed with the Hanes and Isherwood (1949) reagent and then heated to 80^{o} for 10 minutes. Phosphates were detected by the blue colouration after exposure to ultraviolet light. For the determination of metachromatic activity, polyphosphates were precipitated from the concentrated spore washings with barium acetate (saturated solution in acetic acid). The precipitate was washed, resuspended in 5 ml of water and shaken with Amberlite IR-120 (H^{+} form). The barium-free solution was examined for metachromasy with 0.006% (w/v) toluidine blue

(Nassery, 1969) using a Unicam S.P.600 spectrophotometer. The values of E_{530}/E_{630} were then calculated.

Sugars

Sugars in the soluble surface material were examined both before and after hydrolysis in 0.5M sulphuric acid for 12 hours at 105° in a sealed tube. Excess sulphate was removed by precipitation with barium hydroxide and the supernatant solution passed down columns of Amberlite IR-120 (H^+ form) and IRA-400 (Acetate form). Total reducing sugars were determined by the arsenomolybdate method (Chan and Cain, 1966). Individual monosaccharides were identified by paper chromatography. The solvent system was 2-methyl propanol:acetic acid: water (70:2:28 by vol.) and descending chromatograms were run for up to 60 hours to obtain efficient separation. Sugars were detected by spraying the paper with 3% p-anisidine hydrochloride in acetone (Hough, Jones and Wadman, 1950). Colours were developed by heating at 105° for 1 hour and compared with standards under ultraviolet light.

Surface protein

Surface protein was extracted from spores by incubating with 7M urea adjusted to pH 2.8 containing 10% (v/v) 2-mercapto-ethanol for 1 hour at 37°. The suspension was cooled and centrifuged, and the spores washed four times with water and then incubated with 0.1M sodium hydroxide for 15 minutes at 4°. The alkaline extract was dialysed against running water overnight (Gould, Stubbs and King, 1970). Amino acids in the extracted non-dialysable wall material were determined on a Technicon TSM.1 amino acid analyser after hydrolysis with

6M HCl at 100° in an atmosphere of nitrogen for 21 hours.

Total Protein

Total protein was determined using the Folin phenol reagent (Lowry, Rosebrough, Farr and Randall, 1951) after treatment of the walls with 2M NaOH at 100° for 30 minutes (Shah and Knight, 1968).

Detection of cutin acids

Washed spore walls were refluxed with 1% ethanolic potassium hydroxide for 3 hours. After acidification of the hydrolysate with hydrochloric acid organic acids were extracted with ether, methylated with diazomethane, and examined by thin-layer chromatography on Silica Gel HR (Merk) using chloroform: ethyl acetate (7:3 v/v) as solvent. Comparisons were made with the following standards representative of the methyl esters of typical cutin acids - methyl hexadecanoate, methyl 16-hydroxy-hexadecanoate, methyl 10,16-dihydroxyoctadecanoate and methyl threo-9,10,18-trihydroxyoctadecanoate. Compounds were detected by spraying the plate with chlorosulphonic acid and heating, as described in Part II (page 64). Gas-liquid chromatography was carried out using a Hewlett-Packard 5750 gas chromatogram as previously described (page 64). Retention data were determined on E301 (5% loading) on Chromosorb W AW DMCS. The carrier gas was nitrogen, flow rate 35 ml/minute (50 p.s.i.). Analyses were made using temperature programming at a rate of 4° per minute from 150-300° (Baker and Holloway, 1970). Relative retention times of the peaks were determined using n-tricosane as a standard. Relative peak areas were measured from the chart by the triangulation method.

Freeze-etching

Conidia were suspended in 15% glycerol for one hour, centrifuged into a pellet, frozen in liquid Freon 12 at -150° and then subjected to a process of fracturing, etching and coating (Moor, 1966). Platinum-carbon replicas were prepared in a Balzers freeze-etching plant BA 360M and viewed in an AEI EM6B electron microscope.

Scanning electron microscopy

Spores were attached to metal specimen holders with "Durafix" adhesive and, after coating under vacuum with gold/palladium, viewed in a Stereoscan electron microscope.

RESULTS

Electrophoretic properties of Penicillium conidia

The pH/mobility curves of conidia of five species of Penicillium, harvested from malt agar after seven days growth, were all different and characteristic (Figure 25). The mobilities of two strains of Penicillium notatum were similar above pH 6 but diverged at low pH(Table 8). The curves were affected neither by repeated reculturing of the fungi nor by storage of conidia in water at 0° for up to 2 days. There was no evidence to suggest that suspensions of conidia in the acid or alkaline buffer caused any surface denaturation; after resuspension in pH 7.0 buffer mobilities were the same as those of normal control spores. A single washing in the appropriate buffer before mobilities were determined was necessary to ensure complete removal of water from the spores, but this washing had no effect on mobility; spores shaken dry from culture plates had the same mobility as spores harvested by the normal procedure. Electrophoretic mobilities of the virus-

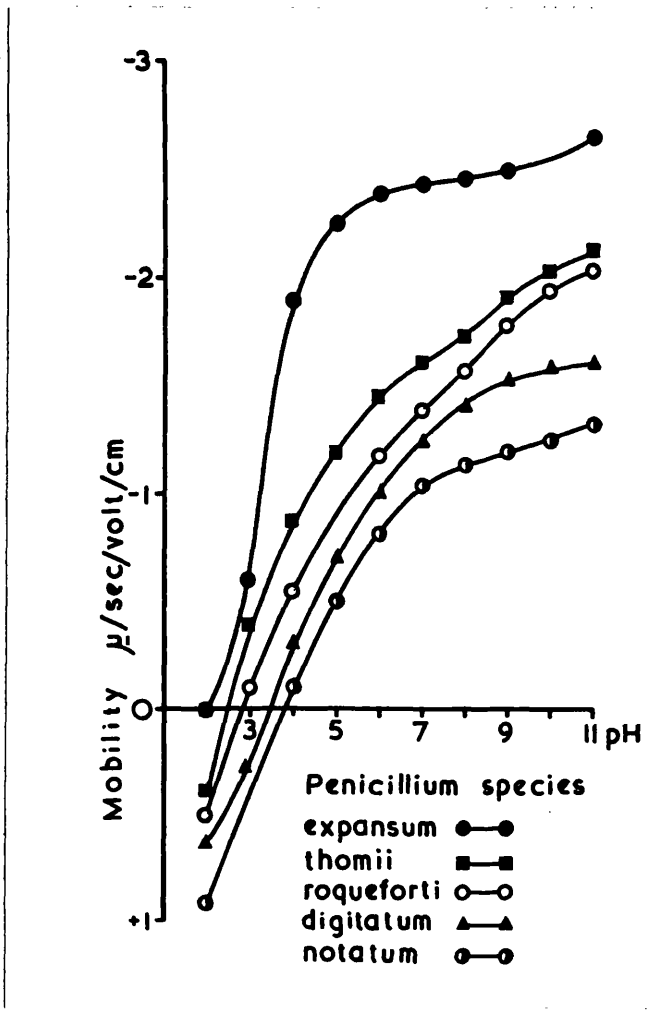


Figure 25. pH/mobility curves of conidia of 5 Penicillium species grown on malt agar.

Table 8. Electrophoretic mobilities of conidia of two strains of Penicillium notatum.

pH	Mobilities (µ/sec/volt/cm)	
	Penicillium notatum C.M.I. 15378	Penicillium notatum C.M.I. 17969
2.0	+ 0.10	+ 0.87
3.0	- 0.26	+ 0.32
5.0	- 0.65	- 0.52
7.0	- 0.93	- 1.00
9.0	- 1.16	- 1.22
11.0	- 1.38	- 1.34

infected strain of Penicillium stoloniferum (ATCC 14586) and the corresponding virus-free isolate (ATCC 14586 B3/2) were very similar (Table 9). Replicas of freeze-etched conidia of the virus free strain are shown in Plates 25 and 26. No differences were observed in surface detail of normal and virus-infected spores.

Table 9. Electrophoretic mobilities of conidia of virus free and infected strains of Penicillium stoloniferum.

	Electrophoretic mobility μ/sec/volt/cm	
pH	Penicillium stoloniferum Virus-free (ATCC 14586 B3/2)	Penicillium stoloniferum Virus-infected (ATCC 14586)
2.0	+ 0.66	+ 0.75
4.0	- 0.56	- 0.32
6.0	- 1.05	- 0.90
8.0	- 1.58	- 1.34
10.0	- 1.66	- 1.47

The effect of age on the pH/mobility curve of conidia of Penicillium expansum

The pH/mobility curve of 1-day old conidia grown on malt agar has a typical amino-carboxyl shape with an isopotential point at pH 3.5 (Figure 26). After two days the pH/mobility curve shows an acid surface with an isopotential point of 2.0 closely resembling that from 7-day old conidia (Figure 27). As the shape of the pH/mobility curve showed little further change after 2 days, all subsequent tests were carried out on spores harvested from 7-day old cultures.

The electrokinetic properties of Penicillium expansum conidia

The pH/mobility curve for Penicillium expansum conidia grown on malt agar (Figure 27) with an isopotential point at pH 2.0 suggests the presence of highly acidic phosphate groups. Treatment with alkaline phosphatase revealed an underlying

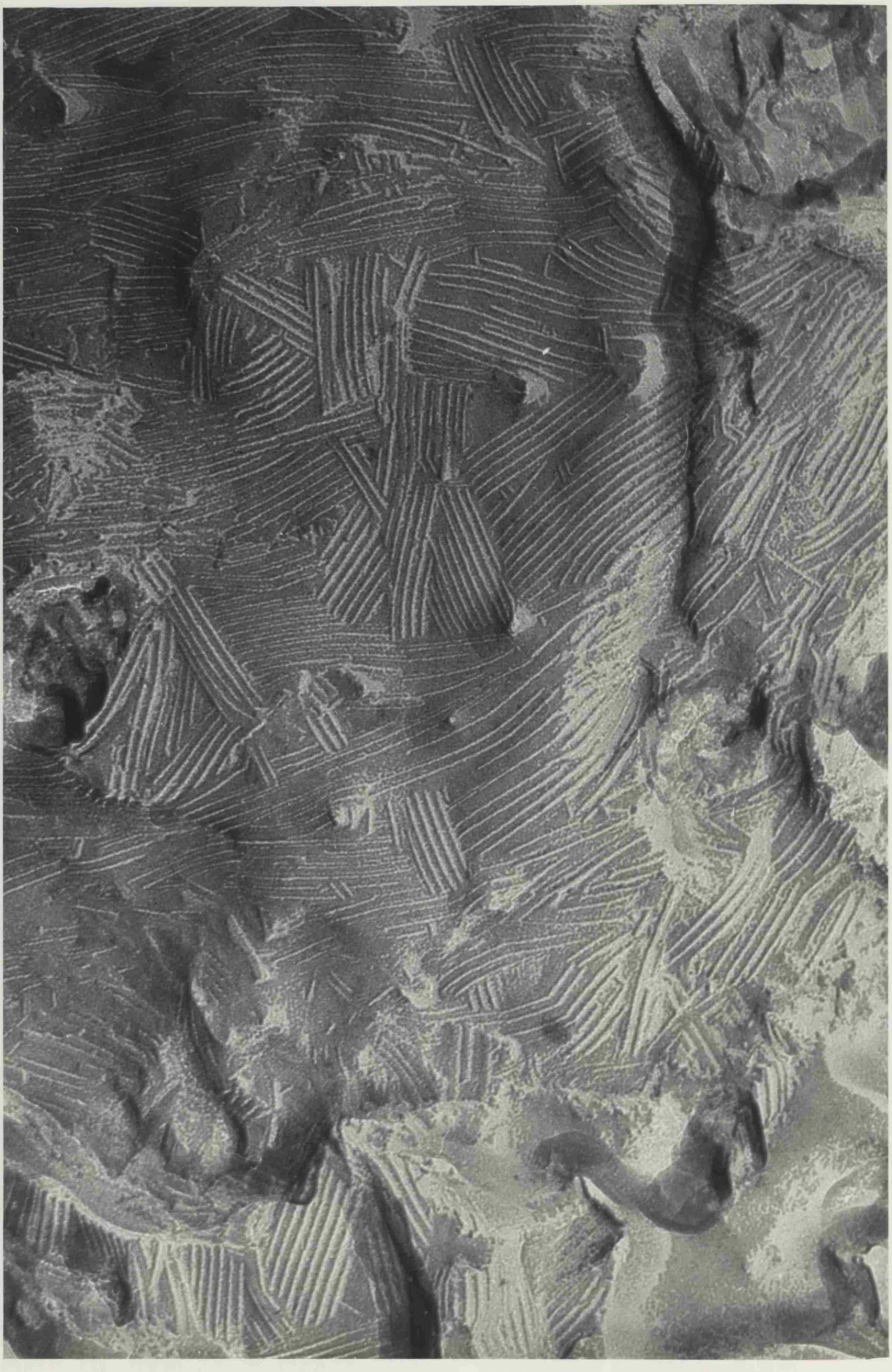


Plate 25. Surface replica of freeze-etched conidium of Penicillium stoloniferum (virus-free strain) X 125,000.

Plate 26. Replica of freeze-etched conidium of Penicillium stoloniferum (virus-free strain) showing plasma membrane and cell wall X 40,000.

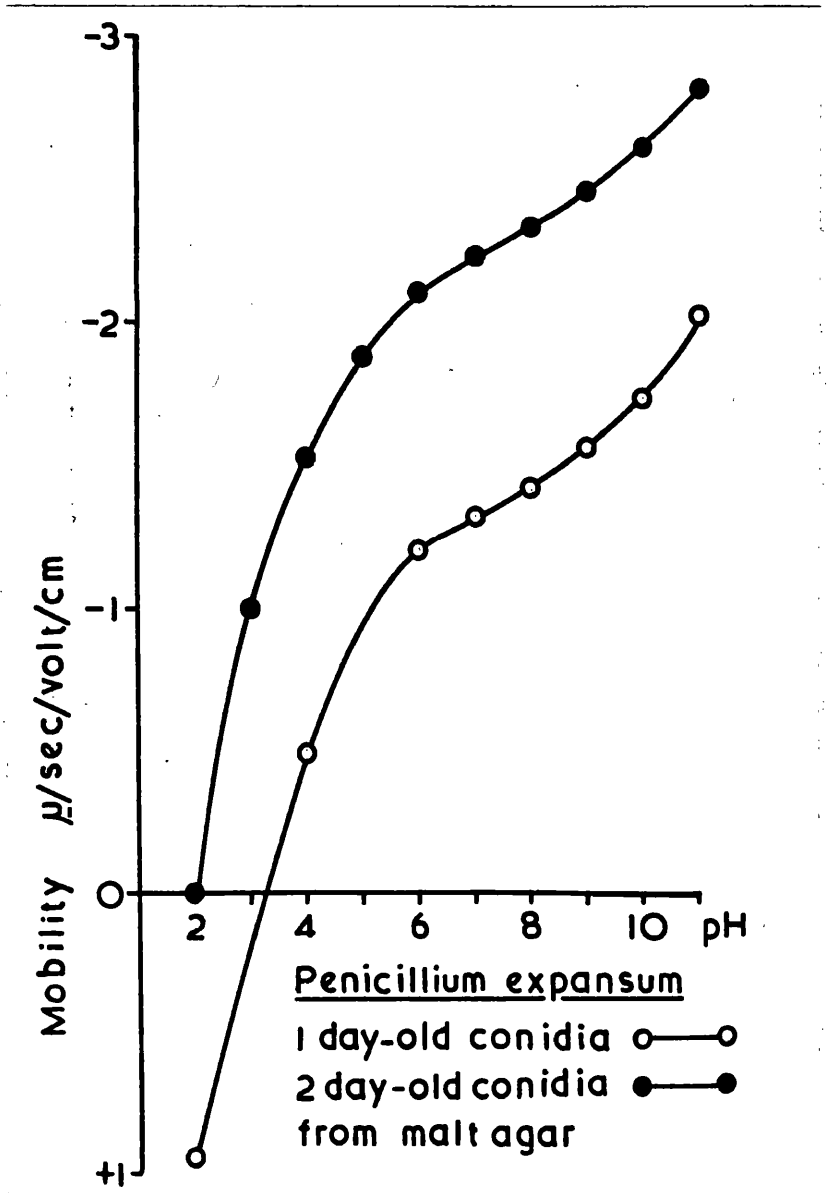


Figure 26. Variation in pH/mobility curve with age of conidia.

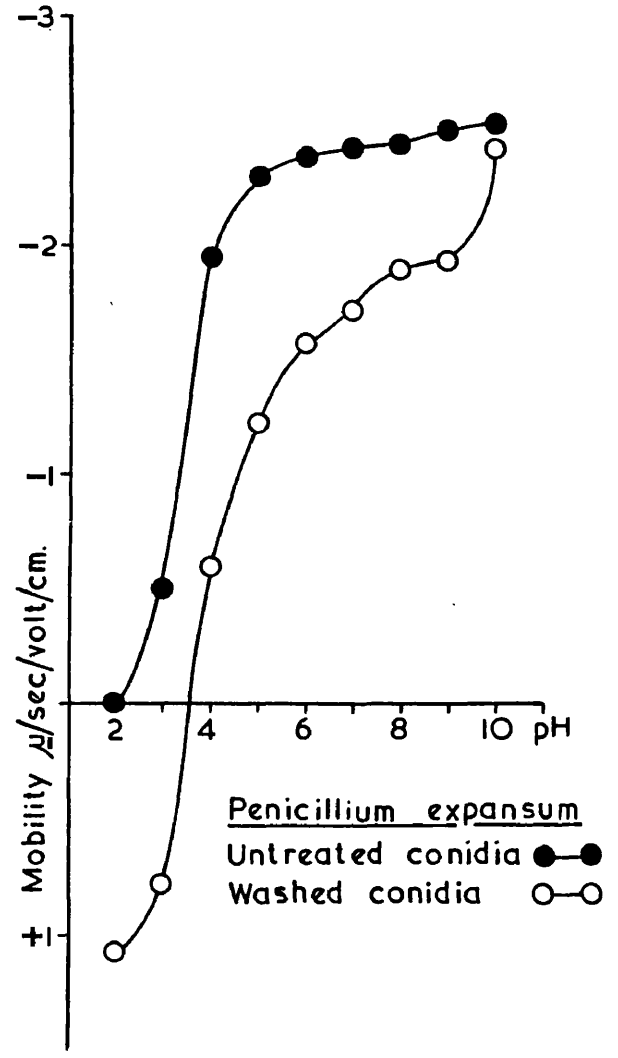


Figure 27. Penicillium expansum: pH/mobility curve of untreated conidia and of conidia washed 10 times with 10% (w/v) sucrose, 5 times with 0.9% (w/v) NaCl and 5 times with water.

amino-carboxyl surface with an isopotential point of 3.5 (Figure 28). Confirmation of the presence of phosphate groups on intact P.expansum spores was provided by the decrease in mobility of conidia suspended in barbiturate buffer (pH 7.0,I:0.05) from -2.40 to -1.60 μ/sec/volt/cm in the presence of 0.01M Ca^{2+}. This method has been used to demonstrate the presence of surface phosphate groups on ascite tumour cells (Forrester et al., 1965). The isopotential point of spores washed in light petroleum or

chloroform:methanol (2:1 v/v) at room temperature for one minute was unchanged indicating that phosphates were not present as phospholipid. Conidia of P.expansum treated with diazomethane (Figure 28) showed decreased negative mobility due to removal of the charge on the carboxyl groups; after hydrolysis of the methyl ester the mobility was still lower than that of the untreated conidia, suggesting that DAM treatment had some blocking action on the amino groups. Interpretation of the pH/mobility curve obtained from FDNB-treated conidia was complicated by the simultaneous removal of some of the labile phosphate component.

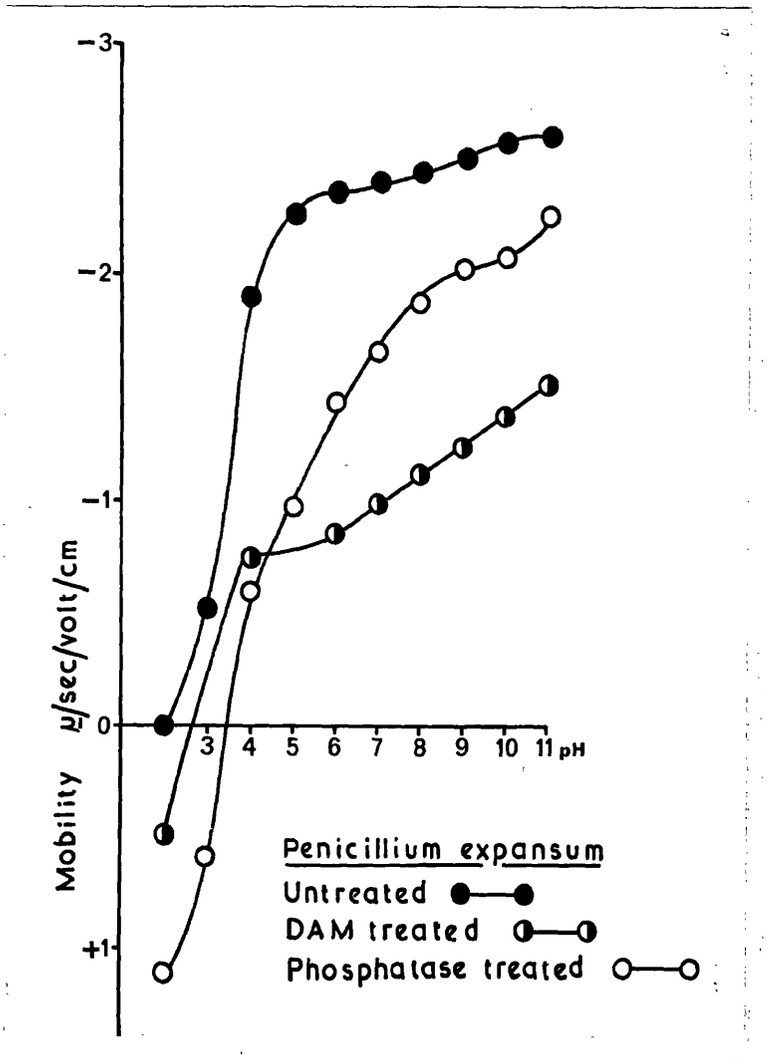


Figure 28. pH/mobility curves of normal and modified Penicillium expansum conidia.

The influence of growth medium composition on the pH/mobility curve of Penicillium expansum conidia

The pH/mobility curve of conidia grown on Fries medium containing 3.0 g KH_2PO_4 per litre (Figure 29) was quite different from that of conidia grown on malt agar. An amino-carboxyl surface was indicated. Conidia harvested from Fries medium containing 0.03 g KH_2PO_4 per litre had a pH/mobility curve of similar shape although mobilities were higher above pH 7.0.

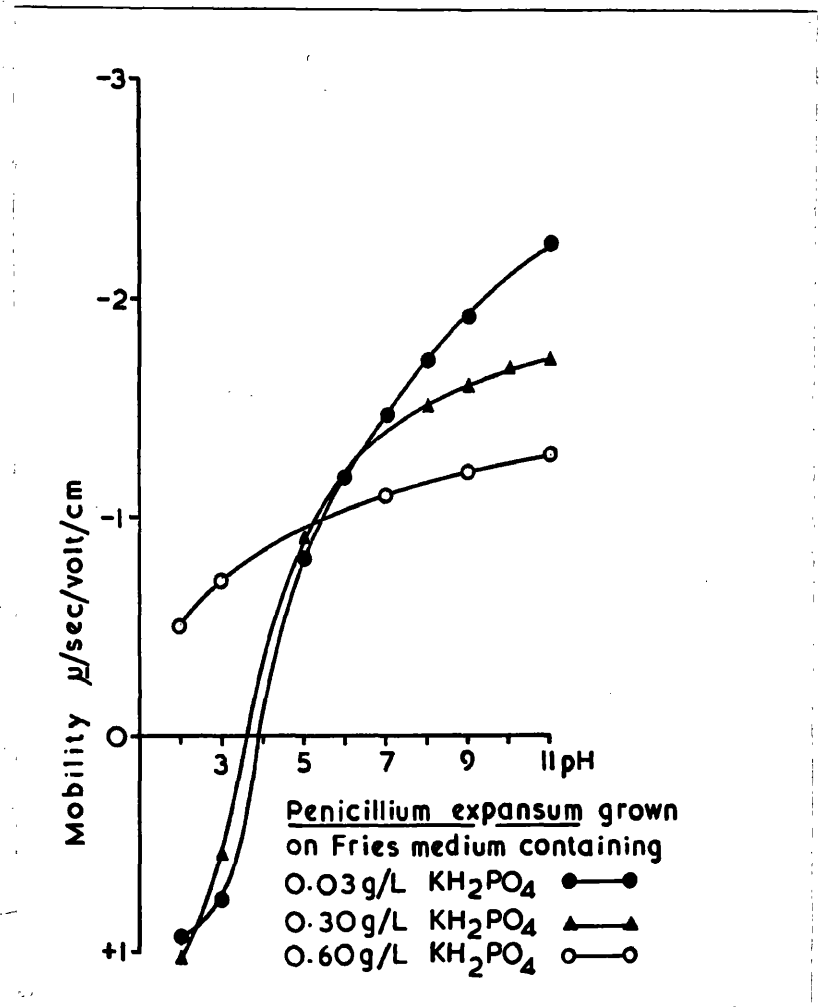


Figure 29. The effect of the composition of culture medium on the pH/mobility curve of Penicillium expansum conidia.

When the phosphate content of the medium was increased to 6.0 g KH_2PO_4 per litre, conidia had a completely acid surface (Figure 29). Spores shaken dry from culture plates had the same mobility as spores harvested by washing from the surface with a jet of water showing that surface phosphate was originally present and had not been adsorbed from the growth medium.

The effect of washing on the spore surface

The surface of conidia grown on malt agar became progressively less acid as the conidia were washed (Table 10).

Table 10. Electrophoretic mobilities of washed conidia of Penicillium expansum grown on malt agar.

Washing procedure	Isopotential point (pH)	Electrophoretic mobilities μ/sec/volt/cm			
		pH 2.0	pH 3.0	pH 4.0	pH 5.0
Unwashed (control)	2.0	0.00	-0.60	-1.87	-2.23
Water (x 5)	3.4	+0.25	+0.08	-0.15	-0.36
Water (x 10)	3.5	+0.55	+0.24	-0.21	-0.42
Water (x 15)	3.6	+0.63	+0.37	-0.33	-0.71
10% Sucrose (x 10) 0.9% NaCl (x 5) Water (x 5)	3.7	+1.08	+0.77	-0.55	-1.10

The washing technique of Dyke (1964), was as effective as alkaline phosphatase in removing surface phosphate groups (Figure 27). The rapid increase in isopotential point from 2.0 to 3.4 after only five washings showed that most surface phosphate is easily removed. Further washings produced only a small increase in isopotential point, but the large increase in positive mobility at pH 2.0 and the similar negative increase at pH 5.0 suggests that prolonged washing removed non-ionogenic material. Extensive washing by Dyke's technique revealed a

typical amino-carboxyl surface. Treatment of washed spores with 1-fluoro-2,4-dinitrobenzene (FDNB) increased the negative mobility (Figure 30) confirming the presence of amino groups.

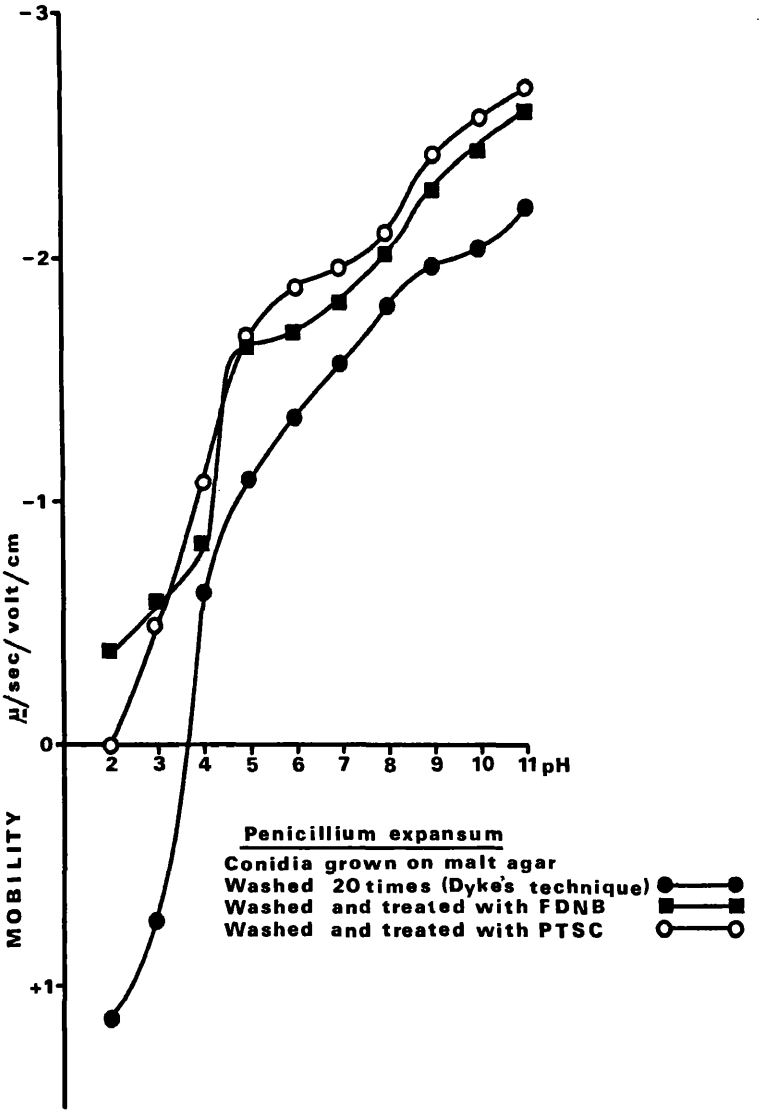


Figure 30. The effect of washing and of modification of surface amino groups on the pH/mobility curve of Penicillium expansum conidia.

The inflexion at pH 7 to pH 8 was not due to secondary amino groups as it was still present when washed conidia were treated with p-toluenesulphonyl chloride (Figure 30), a reagent which reacts with both primary and secondary amines (Gittens and James, 1963). Hydrolysis of FDNB-treated conidia followed by chromatographic examination using the methods described in

part 1 (page 32) failed to indicate the presence of any DNP-amino acids. A compound not definitely identified, with an R_F value similar to that of the DNP derivatives of D-glucosamine and D-galactosamine was however present. These results show that no free amino groups are present on the surface protein and indicate that surface polysaccharides are also present on the washed spore. When conidia grown on Fries medium with the higher phosphate content were subjected to prolonged washing the acid surface was only partially removed, showing that the acid groups have become an integral part of the surface.

The composition of spore washings

The amount of phosphorus compounds washed from conidia of Penicillium expansum varied widely with the nature and phosphorus content of the medium (Table 11). The small amount of phosphorus removed from conidia grown on Fries medium with the highest phosphate content is in agreement with the electrophoretic results which showed that even prolonged washing had little effect on the acid surface (Figure 31). Polyphosphates react with toluidine blue causing a hypsochromic shift of the absorption peak (Wiame, 1947). Under controlled conditions this reaction is specific (Damle and Krishnan, 1954). The metachromatic activity of the washings established that polyphosphates were present on all spore surfaces. The highest polyphosphate content occurred on the surface of spores grown on malt agar but small amounts were present on conidia grown on Fries medium. Conidial production was greater on malt agar than on Fries medium even when the phosphorus contents of the two media were similar. Thin layer and paper chromatography

Table 11. The effect of growth medium on the phosphorus content of conidia and conidial washings from Penicillium expansum.

Conidia were grown, harvested and washed as described in the text.

Medium	Phosphorus g/l	Dry weight of conidia[1] mg	mg/g dry weight of conidia: Phosphorus in conidia	mg/g dry weight of conidia: Phosphorus in conidial washings	Phosphorus removed by washing %	Metachromatic activity of washing[2]
Malt agar	1.33	810	10.7	0.15	1.4	0.17
Fries	0.07	74	9.8	-	-	0.05
"	0.70	584	9.3	0.07	0.8	0.04
"	1.40	561	9.3	0.04	0.4	0.03

1 From 50 plates

2 E_{530}/E_{630} mμ - blank

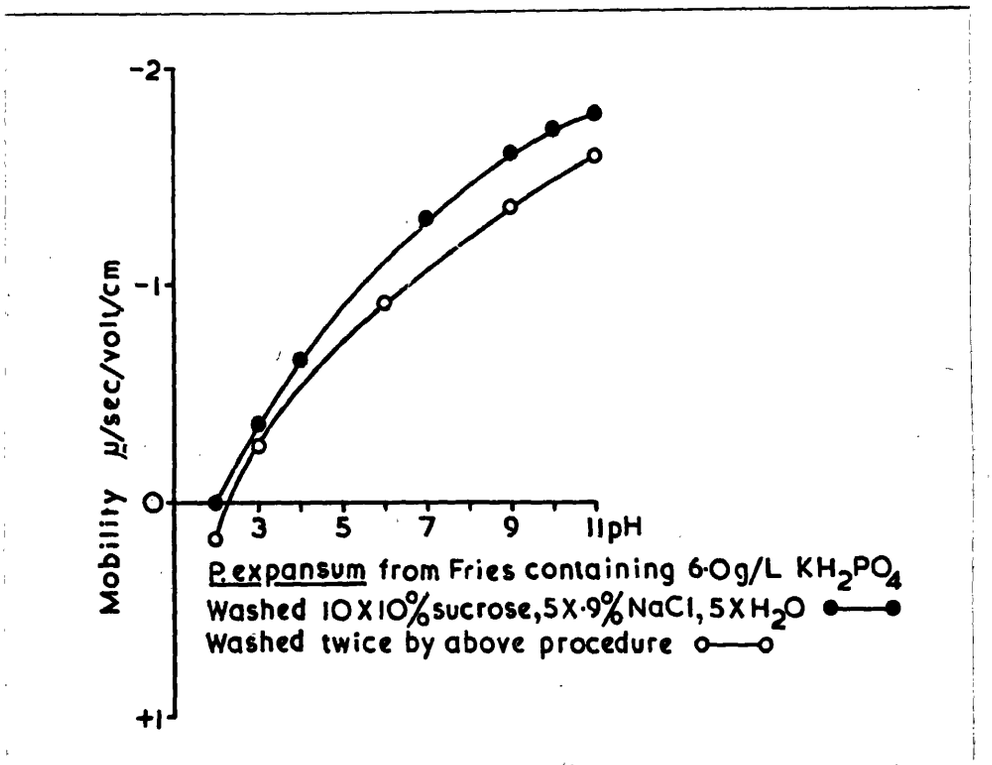


Figure 31. The effect of washing on the pH/mobility curve of Penicillium expansum conidia grown on "high phosphate" medium.

of the concentrated water washings from conidia grown on malt agar confirmed the presence of polyphosphates. Discrete separations were not obtained but the presence of compounds with chain lengths containing less than ten phosphorus atoms was demonstrated. The extracted material had no absorption at 260 mμ showing that nucleic acids were not present. Imidophosphate linkages were also absent since no infrared absorption peak occurred at 1400 cm^{-1} (Correll, 1966).

As the prolonged washing of conidia grown on malt agar seemed to remove non-ionogenic material (Table 10) the water washings were examined for carbohydrates. The total reducing sugars present in twenty successive water washes, expressed

as glucose, amounted to 7.1 µg/gram dry weight of spores before hydrolysis and 12.6 µg/gram dry weight after hydrolysis. Glucose and xylose were identified before hydrolysis and, in addition, arabinose after hydrolysis.

The nature of the surface rodlet layer

Hess et al. (1968) showed the surface of Penicillium conidia to be covered with a distinctive rodlet structure, and suggested that this might consist of cross-linked hydroxy-acid polymers such as cutin or a similar material since the layer was removed by aqueous or ethanolic potassum hydroxide. Penicillium expansum spore walls were extracted with 1% ethanolic potassium hydroxide and examined by thin-layer and gas-liquid chromatography. None of the hydroxy fatty acids characteristic of cutin (Baker and Holloway, 1970) could be detected by either method. The fatty acids extracted from the cell walls consisted mainly of palmitic, oleic and stearic acids although five other acids were present in small amounts (Table 12).

Table 12. The relative composition of fatty acids extracted by 1% alcoholic potassium hydroxide from washed spore walls of Penicillium expansum (methylated and chromatographed on 5% E301 programmed 150 - 300°).

T_{rel} tricosane	Relative composition	Identity
0.18	1.25	C_{12}
0.34	5.50	C_{14} 1Δ
0.45	2.20	
0.55	46.50	C_{16}
0.65	3.13	
0.68	1.95	
0.75	19.93	C_{18} 1Δ
0.79	19.54	C_{18}

1Δ = mono unsaturated

The scanning electron microscope showed the typical chain-like arrangement of Penicillium expansum spores but disclosed little surface detail (Plates 27 and 28). Examination of replicas obtained by freeze-etching (Plates 29, 30, 31 and 32) revealed a pattern of rodlets similar to those shown to be present on spores of other Penicillium species (Hess et al., 1968) The rodlet structure closely resembles

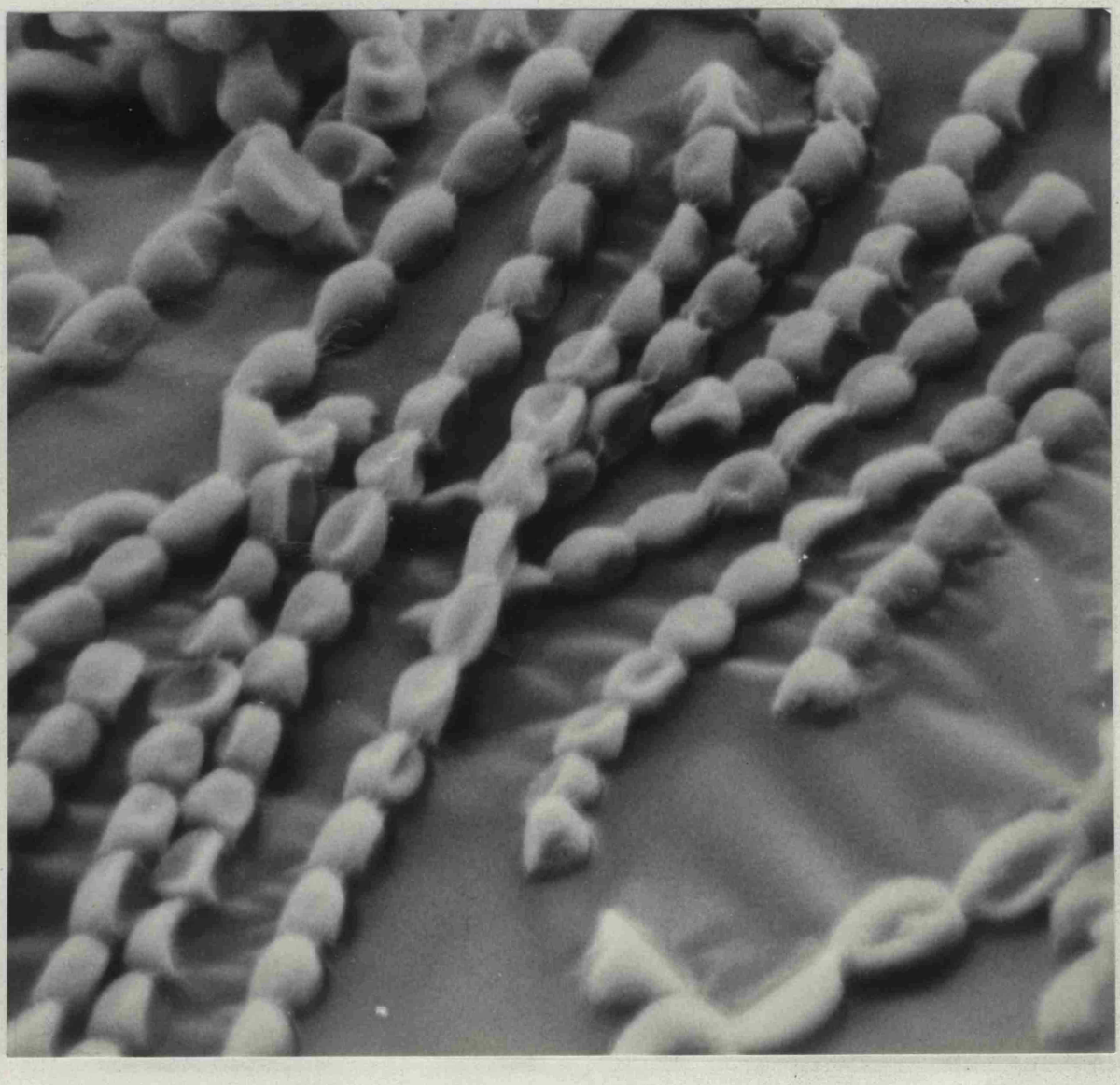


Plate 27. Penicillium expansum conidia
X 2500 (Stereoscan).

the patterns observed on some bacterial spores (Holt and Leadbetter, 1969). The patterned surface layer on Bacillus coagulans spores consists of an alkali-soluble protein which can be removed from the spores by treatments which rupture disulphide bonds (Gould et al., 1970). Surface rodlets of Penicillium expansum were not removed by incubation of the spores with mercaptoethanol in urea followed by treatment with

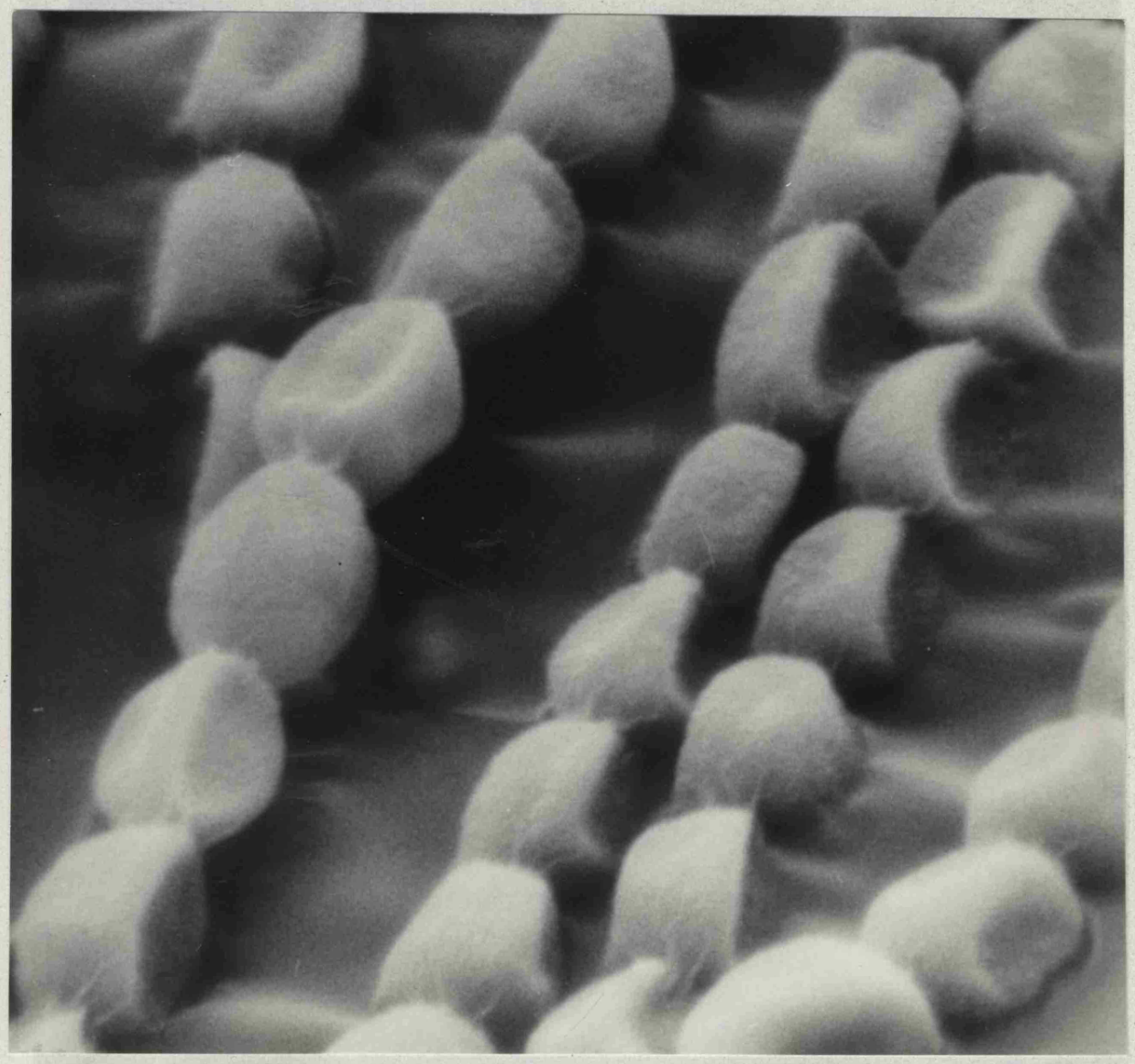


Plate 28. Penicillium expansum conidia X 7500 (Stereoscan).

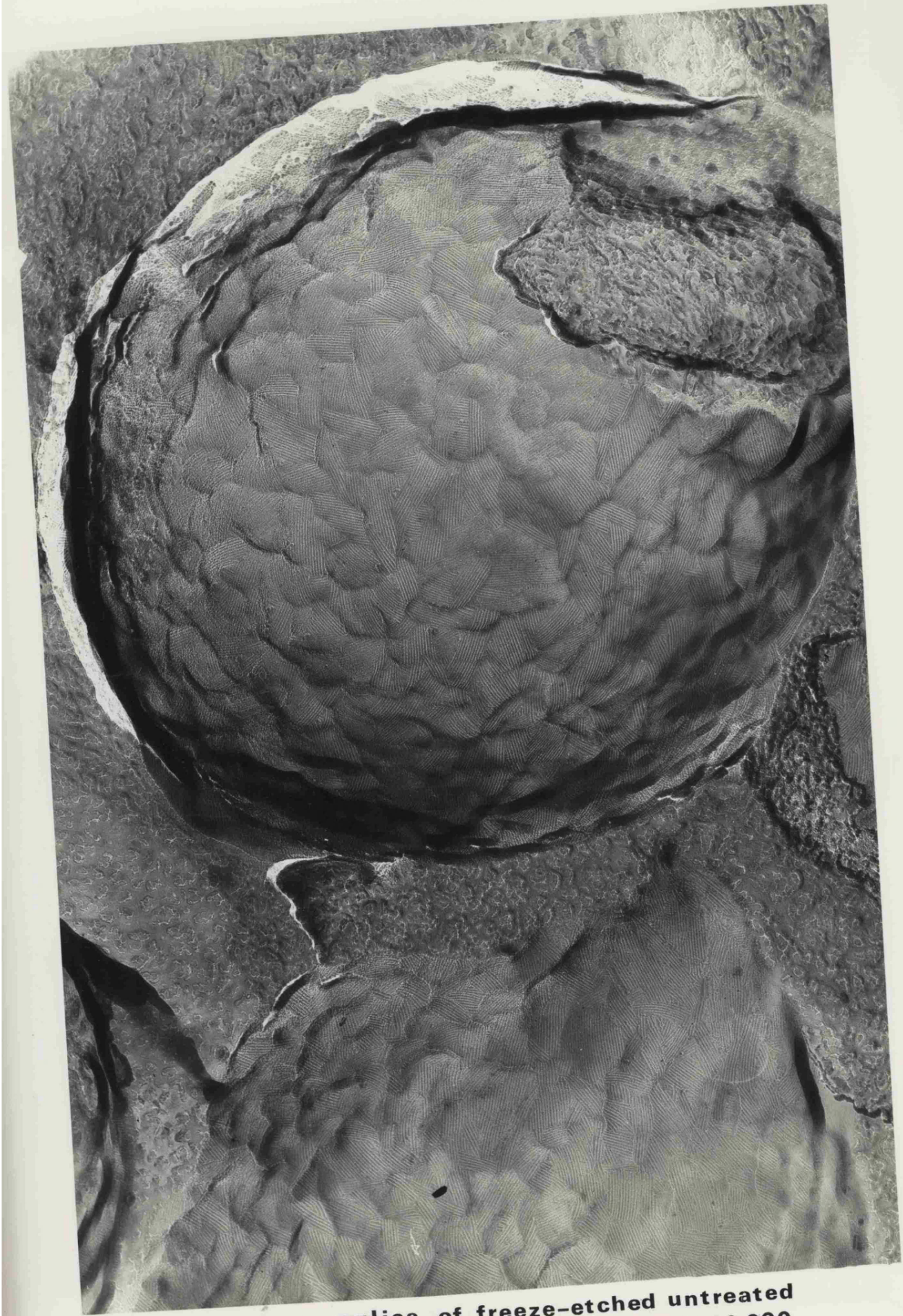


Plate 29. Surface replica of freeze-etched untreated conidium of Penicillium expansum X 40,000.

Plate 30. Surface replica of freeze-etched untreated conidium of Penicillium expansum X 80,000.

Plate 31. Surface replica of freeze-etched untreated conidium of Penicillium expansum X 120,000.

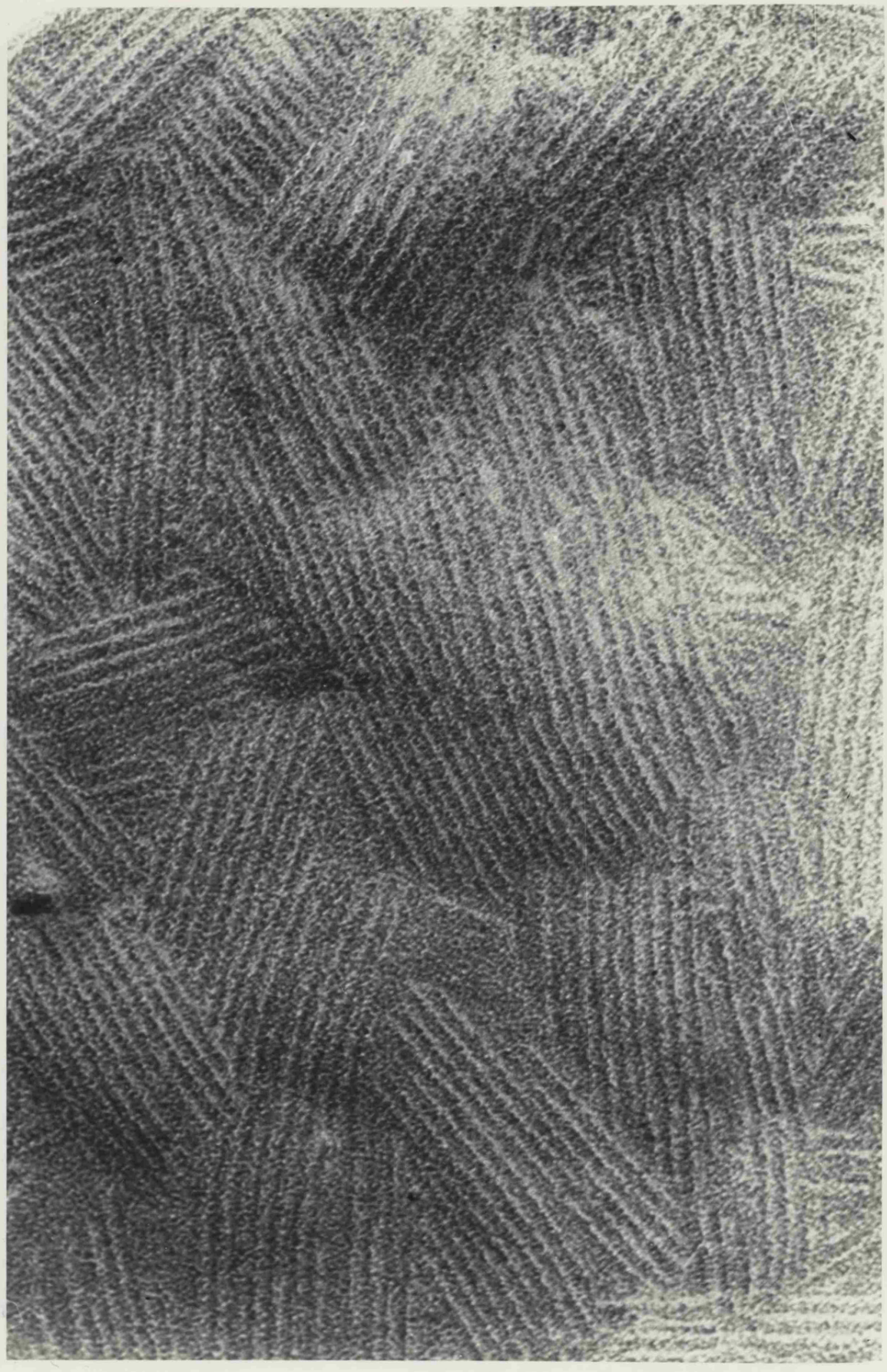


Plate 32. Surface replica of freeze-etched untreated conidium of Penicillium expansum X 200,000.

0.1M sodium hydroxide. The rodlets were however less distinct after alkali treatment (Plates 33 and 34) suggesting that some protein material may have been removed. The electrophoretic mobility of water-washed conidia fell, after alkali treatment from -2.00 to -1.16 μ/sec/volt/cm at pH 10.0 and from 1.10 to 0.64 μ/sec/volt/cm at pH 2.0. The isopotential point was the same in control and treated spores. These decreases in mobility confirmed that the treatment may have partially removed some protein material. Analysis of the non-dialysable alkali-soluble material showed it to contain 11.2% protein. The remainder of the extracted material probably consisted of polymeric polysaccharides (Grisaro, Sharon and Barkai-Golan, 1968). The extracted protein represents only a small proportion of the total cell wall protein (Table 13).

Table 13. Some components of conidial walls of Penicillium expansum grown on malt agar.

	%
Protein	7.67
Phosphorus	0.23
Nitrogen (by Kjeldahl)	3.32
Alkali-soluble surface protein	0.37
Other alkali-soluble material	2.92

Amino acid analyses of hydrolysed proteins from the surface extract, the total cell wall, and the protoplasm of Penicillium expansum conidia are compared in Table 14. In contrast with the cell wall the extracted protein has a high tyrosine and methionine content, proline is absent, whilst smaller amounts of threonine, leucine, isoleucine, histidine, valine and cyst(e)ine are present. The distinctive amino acid composition of the extracted protein confirms the partial removal of a definite surface layer. The total wall protein is similar in

Plate 33. Surface replica of freeze-etched conidium of *Penicillium expansum* after treatment with 10%(v/v) 2-mercaptoethanol in 7M urea followed by 0·1M sodium hydroxide X 40,000.

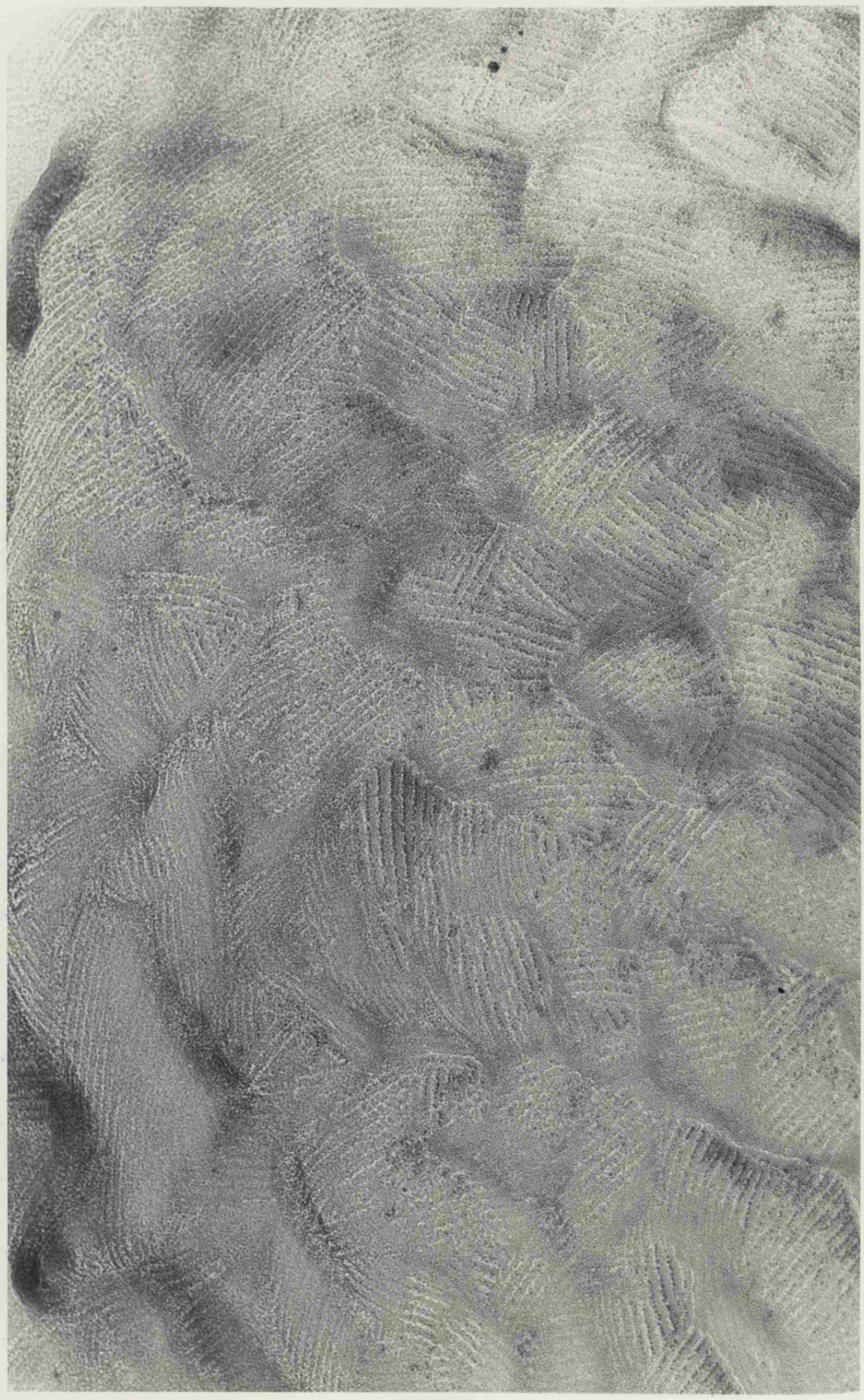


Plate 34. Surface replica of freeze-etched conidium of *Penicillium expansum* after treatment with 10% (v/v) 2-mercaptoethanol in 7M urea followed by 0·1M sodium hydroxide X 100,000.

Table 14. Amino acid components of Penicillium expansum conidia

Amino acid	Mole ratio		
	Alkaline extract from conidia	Conidial wall	Protoplasm
Aspartic acid	1.38	1.25	1.24
Glutamic acid	1.48	1.13	1.11
Threonine	0.50	1.07	0.84
Serine	1.00	1.10	1.14
Alanine	1.00	1.00	1.00
Glycine	1.13	1.02	0.85
Leucine	0.49	0.76	0.74
Phenylalanine	0.40	0.31	0.40
Lysine	0.26	0.31	0.20
Isoleucine	0.18	0.39	0.23
Arginine	0.43	0.50	0.48
Tyrosine	1.09	0.31	0.36
Proline	not detected	0.77	0.51
Histidine	0.30	0.61	0.53
Valine	0.34	0.72	0.48
½ Cystine	0.23	1.30	1.78
Methionine	0.86	0.13	0.13

composition to the protoplasmic protein. Mercaptoethanol pre-treatment had little effect on the removal of surface protein. Surface rodlets were similar in appearance after alkali extraction whether mercaptoethanol was used or not (Plate 35) and the amino acid composition of the extracts were almost identical.

Discussion

The pH/mobility curves of Penicillium conidia are reproducible and characteristic of the species provided the fungi are grown on defined media. The widely differing mobility curves of closely related species can probably be explained by the presence of a polyphosphate layer which is not an integral part of the conidial surface. The mobilities of two strains of Penicillium notatum were shown to be similar above pH 6 but diverged at low pH. Although Penicillium species can be distinguished microscopically, pH/mobility curves may be useful in confirmation of identity and may prove of value in differentiating between sub-species and strains. Cell walls of virus-infected strains of Penicillium stoloniferum contain more galactosamine than those of virus-free isolates (Buck, Chain and Darbyshire, 1969) but the mobilities of conidia of an infected and corresponding normal strain are similar. This suggests that ionogenic surface groups are not affected.

The presence of highly acidic surface phosphate groups on Penicillium expansum conidia grown on malt agar was suggested by the low isopotential point (pH 2.0) and confirmed by treatment with alkaline phosphatase and by the decrease in mobility at pH 7.0 which occurred in the presence of Ca^{2+}. Removal of phosphate either by enzymic action or prolonged washing revealed an underlying amino-carboxyl surface. The curves of

Plate 35. Surface replica of freeze-etched conidium of Penicillium expansum after treatment with 0·1M sodium hydroxide (mercaptoethanol pretreatment omitted) X 125,000.

other Penicillium species are indicative of amino-carboxyl surfaces containing varying amounts of phosphate.

Thin layer chromatography and the metachromatic reaction have established the presence of polyphosphate on conidia of Penicillium expansum. No imidophosphate or nucleic acids were detected in the material removed by washing, and phospholipids are absent since the surface has been shown to be lipid free (Part II). Surface phosphate groups have also be detected on yeast cells (Eddy and Rudin, 1958) and are removed from the surface of Neurospora crassa during the preparation of clean conidial walls, but the exact nature of the phosphate was not determined. Rothstein and Meier (1951) have, however, suggested that uranyl ions may react with polyphosphate-like groups on the yeast surface, and Harold (1962a) has shown that cytoplasmic polyphosphate can bind to hyphal cell walls of N.crassa.

The presence of a three layered wall covered with an outer pattern of rodlets on conidia of Penicillium species has been demonstrated by Sassen, Remsen and Hess (1967). Carbon replica studies suggest that the rodlets are themselves covered with an additional very thin film (Hess et al., 1968). This film may constitute the polyphosphate layer present on unwashed conidia. The pH/mobility curves show that the composition of the polyphosphate film is dependent on the nature and phosphate content of the growth medium; the film is practically absent from conidia grown on a low phosphate medium. Even on malt agar medium the polyphosphate layer is absent from young conidia and only appears 2 days after conidial initiation. Thus the polyphosphate layer is not an integral part of the conidial surface. Eddy and Rudin (1958) found a similar situation in

yeasts; phosphate groups were absent from the surface of cells grown in phosphate-deficient media.

Polyphosphates occur in bacteria (Harold, 1963), blue-green algae (Talpasayi, 1963; Jensen, 1969), higher plants (Miyachi, 1961; Tewari and Singh, 1964; Nassery, 1969) and numerous fungal species (see Kuhl, 1960). Belosersky (1958) claimed that polyphosphates are transported in a low polymer form from mycelium into spores. In the present investigation the appearance of surface polyphosphates after two days growth supports the concept of a gradual build up within the spore. Nishi (1961) in studies of germinating conidia of Aspergillus niger using ^{32}P showed that in early stages of germination there was no incorporation of extracellular phosphorus into spores. Most intracellular phosphorus compounds originated from "reserve phosphorus" contained in the conidia. In micro-organisms polyphosphates are usually considered to occur as intracellular "metachromatic granules" and various functions have been proposed. Polyphosphates are thermodynamically "high energy" phosphate compounds and may act as stores of energy from which phosphoryl groups may be reversibly transferred to ADP forming ATP (Kuhl, 1960). Harold (1962b) in studies of Neurospora crassa showed however that ATP formation was not the major route of polyphosphate utilisation and concluded that polyphosphates may act simply as an inorganic phosphate reserve. Utilisation of surface polyphosphates on Penicillium spores whether as an energy source or as a phosphate reserve would probably be dependent on the existence of extracellular polyphosphatases.

Conidia grown on malt agar have an easily-removable outer phosphate layer and an inner non-ionogenic layer consisting

mainly of carbohydrate. The washing technique removed each layer in sequence. The presence of free xylose in the carbohydrate layer is of interest. Xylose has been found in hyphal cell walls of Penicillium chrysogenum (Hamilton and Knight, 1962), Penicillium digitatum and Penicillium italicum (Grisaro et al., 1968). Rizza and Kornfeld (1969) were however unable to detect xylose in either hyphal or conidial walls of P.chrysogenum. Arabinose, which was found in the carbohydrate layer after hydrolysis, has also been found in small amounts in mycelial walls of Aspergillus niger (Johnston, 1965).

The rodlet layer appears to be an integral part of the wall structure since it is not easily separated from the rest of the wall by mechanical disruption. This layer is cutin-free and is not composed of protein with a characteristic composition. The surface layer does however contain protein of a different amino acid composition from that present in the whole wall. The amino acid composition of the whole conidial wall is similar to that of the hyphal wall of Penicillium notatum (Applegarth, 1967) except for the presence of valine. Conidial walls of Penicillium chrysogenum are however, quite distinct, since tyrosine, phenylalanine, methionine and histidine are absent (Rizza and Kornfeld, 1969). Fungal cell walls have frequently been reported to contain a full complement of amino acids (Crook and Johnston, 1962; Shah and Knight, 1968; Aronson and Fuller, 1969). The high tyrosine and methionine content of the surface protein may be significant. Tyrosine is a precursor of melanin which can protect fungi from enzymic lysis (Kuo and Alexander, 1967; Bull, 1970), while methionine as S-adenosylmethionine is an important methyl donor in plants and micro-organisms (Meister, 1965). The

latter compound is extremely reactive (Shapiro and Schlenk, 1960) and may also serve to detoxify injurious substances. Although the rodlet layer superficially resembles the surface layer of Bacillus coagulans spores (Gould et al., 1970) the two layers are different. The characteristic pattern on the bacterial spores is readily removed by mechanical disruption or treatment with reagents which rupture disulphide bonds. Proteins constitute 60-80% of the material extracted by alkali from the bacterial spores: the alkali-soluble fraction from Penicillium expansum conidia contains only 11.2% protein. Unlike the P.expansum spore surface extract the surface protein from bacterial spores is characterised by exceptionally high levels of acidic and basic amino acids.

Water-repellency can probably be attributed to the rodlet layer. The polyphosphate layer does not contribute materially to the water-repellent properties of spores since conidia grown on low-phosphate media lack polyphosphate and are still water-repellent. The Penicillium spore surface has been shown to be lipid-free (Part II); the present work has not revealed any other substances which could be responsible for water-repellency. The wettability of a surface is governed both by the nature of the exposed chemical groups (Adam and Jessop, 1925) and by the surface roughness (Wenzel, 1936). Roughness may give rise to composite surfaces comprising both solid/liquid and air/liquid interfaces. Wettability is most conveniently measured by means of contact angles which give an inverse measure of the adhesion between a solid and a liquid. Cassie and Baxter (1944) have shown that

$$\cos \theta_2 = f_1 \cos \theta_1 - f_2$$

where θ_1 = the contact angle on a smooth surface (absolute contact angle).

θ_2 = the contact angle on the rough surface (apparent contact angle).

f_1 = the area of liquid/solid contact per unit area.

f_2 = the area of liquid/air contact per unit area.

The apparent contact angle and hence the resistance to wetting, increases as the fraction of liquid/air surface increases. Wetting is thus inhibited by the trapping of air in surface invaginations and the physical conformation of the surface may be sufficient to account for the hydrophobic properties of fungal spores. Similar surface factors have been shown to influence the wetting of leaves of higher plants (Holloway, 1970).

P A R T IV

THE EFFECT OF CATIONIC SURFACE-ACTIVE AGENTS ON THE ELECTROPHORETIC MOBILITY OF FUNGAL CONIDIA

PART IV

THE EFFECT OF CATIONIC SURFACE-ACTIVE AGENTS ON THE ELECTROPHORETIC MOBILITY OF FUNGAL CONIDIA

The preceding investigations have shown a wide variation in the surface components of fungal spores. Most fungal diseases of plants are spread by spores, and protective fungicides act by inhibiting germination at the leaf surface. The micro-electrophoretic technique has been used to study the reaction of toxic surface-active agents at the surface of bacterial cells (James, 1965b). Cationic surface-active agents find use as protective fungicides. In Part IV a study is made of the effect of the fungicides dodine and glyodin on the electrokinetic properties of spores, isolated spore walls, and protoplasts, in order to investigate the influence of surface reactions on fungitoxicity.

The cationic surfactant dodine (n-dodecylguanidine acetate) is widely used in the control of apple scab. It is less effective against other diseases (Byrde, 1969). Dodine is rapidly accumulated by fungi from aqueous solution though the amount and rate of uptake varies (Miller, 1960). Bartz and Mitchell (1970a) concluded that differences in fungistatic action were due in part to the time required for the effective dose to be absorbed. Somers (1963b) showed that almost half the saturation level of dodine was associated with the walls of Neurospora crassa spores. Somers and Pring (1966) suggested that the site of toxic reaction was within the protoplasm and that much of the active material was detoxified by binding to the cell wall. Uptake of dodine by Alternaria tenuis and

N.crassa conidia followed an ionic bonding pattern. Cell walls of A.tenuis had a greater capacity to bind dodine than did those of N.crassa. They concluded that the fungicide altered the permeability of the protoplast membrane thus permitting more fungicide to penetrate to the cytoplasm where it destroyed the intracellular structure, though this may not be the primary mode of action of the compound. Brown and Sisler (1960) found that dodine inhibited intracellular enzymes involved in carbohydrate metabolism of Saccharomyces pastorianus cells, while Bartz and Mitchell (1970b) demonstrated metabolic detoxication of dodine in conidia of Fusarium solani. Glyodin (2-heptadecyl-2-imidazoline acetate) is another cationic surfactant used in the control of apple scab. A similar uptake of this fungicide at wall receptor sites might be expected. Owens and Miller (1957) suggest that glyodin is also detoxified by lipid within the cytoplasm. Toxicity is generally attributed to the effect on synthesis of nucleic acids and proteins (Lukens, 1969). Pass, Nurse and Watt (1962) claimed that a mixture of dodine and glyodin gave as good control of Venturia inaequalis as four times the rate of either substance applied alone. Field trials by Albert and Groves (1965), Connor and Heuberger (1966), and Moore (1966) have confirmed the effect.

Materials and Methods

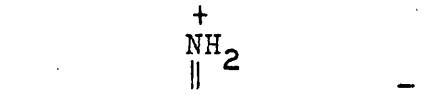
Fungal material

Spores of Alternaria tenuis Nees, Botrytis fabae Sardiña and Neurospora crassa Shear and Dodge macroconidial wild type EM 5297a were grown and harvested as described in Part 1 (page 23). Cell walls of N.crassa were prepared using the Mickle disintegrator and washed free of cytoplasmic contaminants by

the technique of Dyke (1964) as described in Part 1. Protoplasts from young hyphae of N.crassa and from conidia of B.fabae were prepared by incubation with Helix pomatia digestive juice extract as previously indicated (page 25).

Fungicides

Dodine:

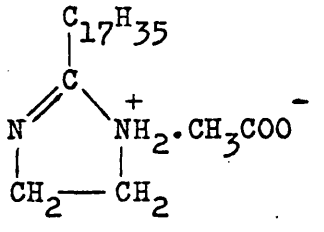


$C_{12}H_{25}$-NH-C($=NH_2^+$)-NH_2.CH_3COO^-

n-dodecylguanidine acetate

A purified sample, melting point 134-135°, was prepared by ethanol-ether recrystallisation from technical grade material. Stock solutions made up in ethanol were diluted in the experimental aqueous solutions so that the final ethanol concentration was less than 2% by volume. This concentration of ethanol had no effect on the electrophoretic mobility or germination of untreated spores.

Glyodin:



2-heptadecyl-2-imidazoline acetate

The compound was recrystallised from iso-propanol. Stock solutions were made up in ethanol and diluted in the experimental aqueous solutions as above.

Microelectrophoresis

The electrophoretic mobilities of conidia and cell walls were measured at a concentration of 1 million/ml in the apparatus described in Part I. The effect of fungicides was measured in sodium acetate buffer of pH 5.6 and ionic strength 0.05. Fungicides were added to the buffered suspensions and electrophoretic observations made within five minutes. Measurements of the electrophoretic mobility of protoplasts

(0.2 million/ml) were made in acetate buffer containing sucrose (0.58M).

Fungitoxicity measurements

The in vitro fungicidal activities were measured by incubating spores with various concentrations of fungicide for 30 minutes at 25°, washing twice, and then diluting to 50,000/ml. Droplets of spore suspensions were incubated on glass slides in a moist chamber at 25° and spore germination recorded after 18 hours. Conidia of Neurospora crassa were diluted and incubated in 0.02% (w/v) sucrose solution in order to obtain a high level of germination of untreated spores. Untreated spores of Alternaria tenuis and Botrytis fabae gave 98-100% germination in distilled water.

Results

The effect of dodine on the electrokinetic properties of spores and isolated spore walls

Changes in the electrophoretic mobility of intact conidia and cell walls of Neurospora crassa on treatment with various concentrations of dodine in acetate buffer (pH 5.6) are shown in Figure 32. The dry weight of cell walls, 8 µg/ml, corresponded to 1 million conidia/ml. The negative charge borne by the spores and cell walls was gradually reduced by the dodine cation until at 580µM for spores and 210µM for walls it was completely neutralised. At higher dodine concentrations the spores became positively charged. Determinations of the fungicidal activity of dodine showed that spores, at 1 million/ml, were completely killed after 30 minutes immersion in solutions of concentrations above 17µM. At this concentration no effect on the mobility of spores was apparent; the ED 50 value was at

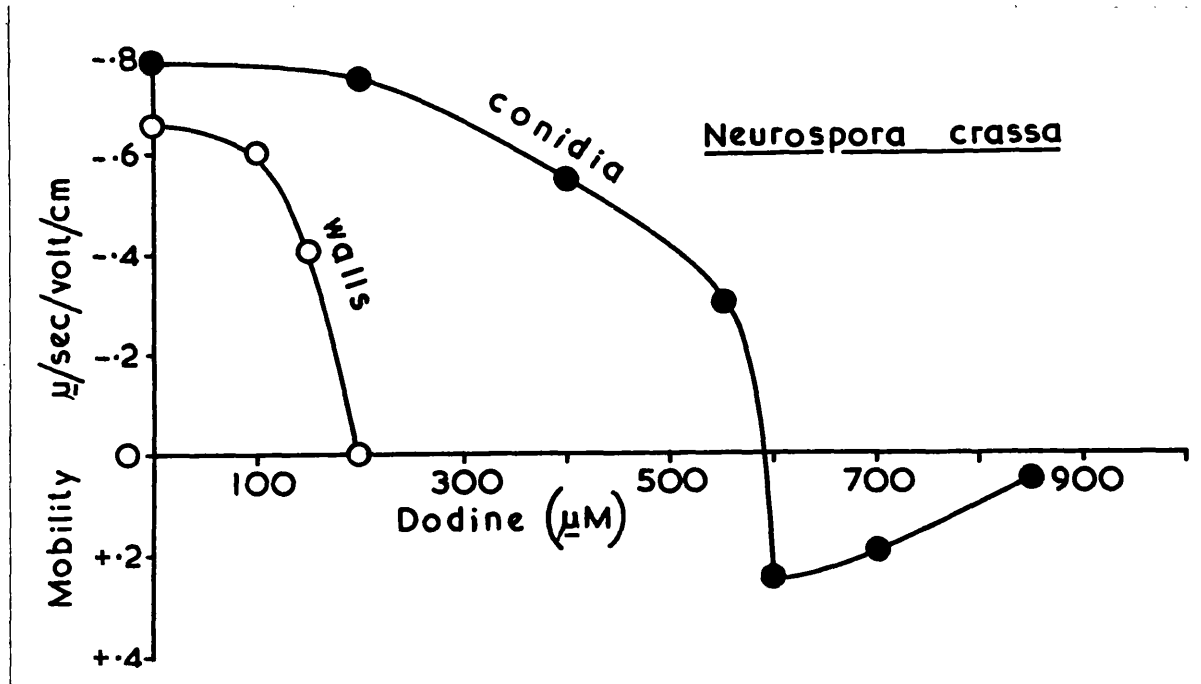


Figure 32. The effect of dodine on the electrophoretic mobility of Neurospora crassa conidia and cell walls in acetate buffer (pH 5.6,I:0.05).

5µM. Spores of Alternaria tenuis showed a linear decrease in mobility in the presence of increasing concentrations of dodine (Figure 33). Charge reversal took place at concentrations of dodine above 190µM. As with N.crassa conidia the fungicidal ED 50 concentration (16µM) had little effect on spore mobility. The uptake of dodine by N.crassa and A.tenuis spores is known to be very rapid (Somers and Pring, 1966), so that the difference in duration between the fungicidal tests (i.e. 30 minutes) and the electrophoretic observations (i.e. up to 5 minutes) is not significant.

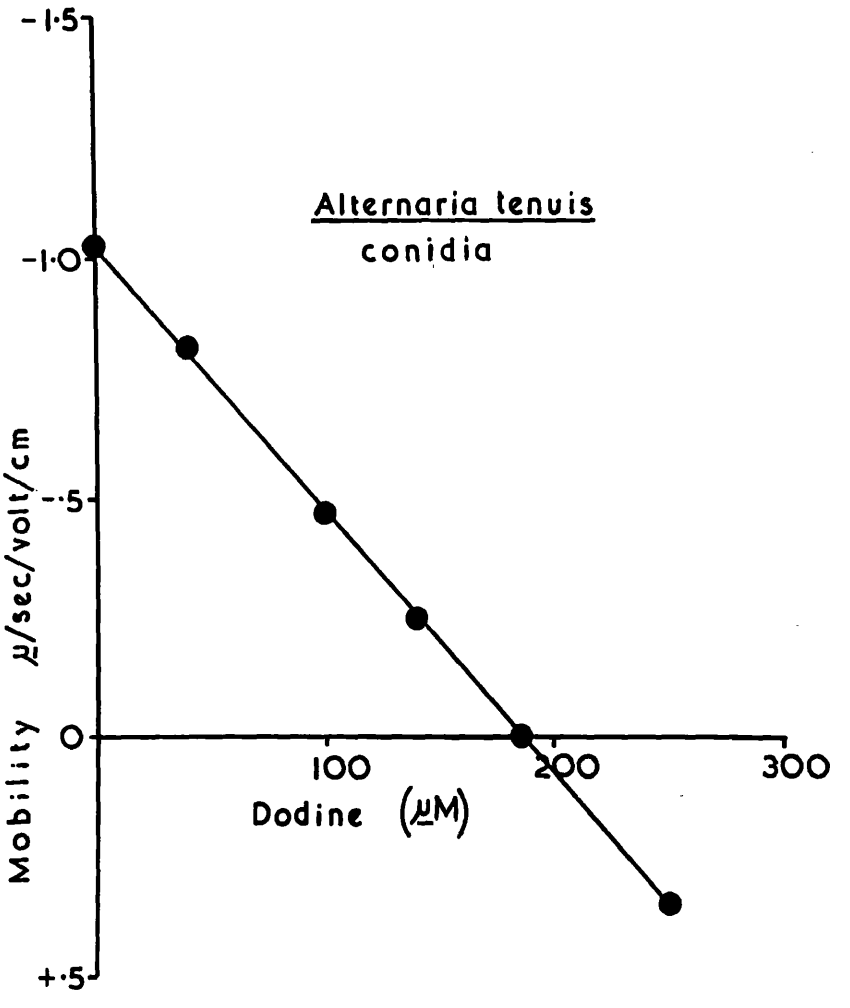


Figure 33. The effect of dodine on the electrophoretic mobility of Alternaria tenuis conidia in acetate buffer (pH 5.6,I:0.05).

The effect of dodine on the electrokinetic properties of protoplasts

The addition of dodine to Neurospora crassa protoplasts stabilised in sucrose-acetate medium caused a rapid reduction in electrophoretic mobility but no evidence of charge reversal (Figure 34). Protoplasts are obviously much more sensitive to the fungicide than conidia, for when conidia at the same concentration, i.e. 0.2 million/ml, were incubated with

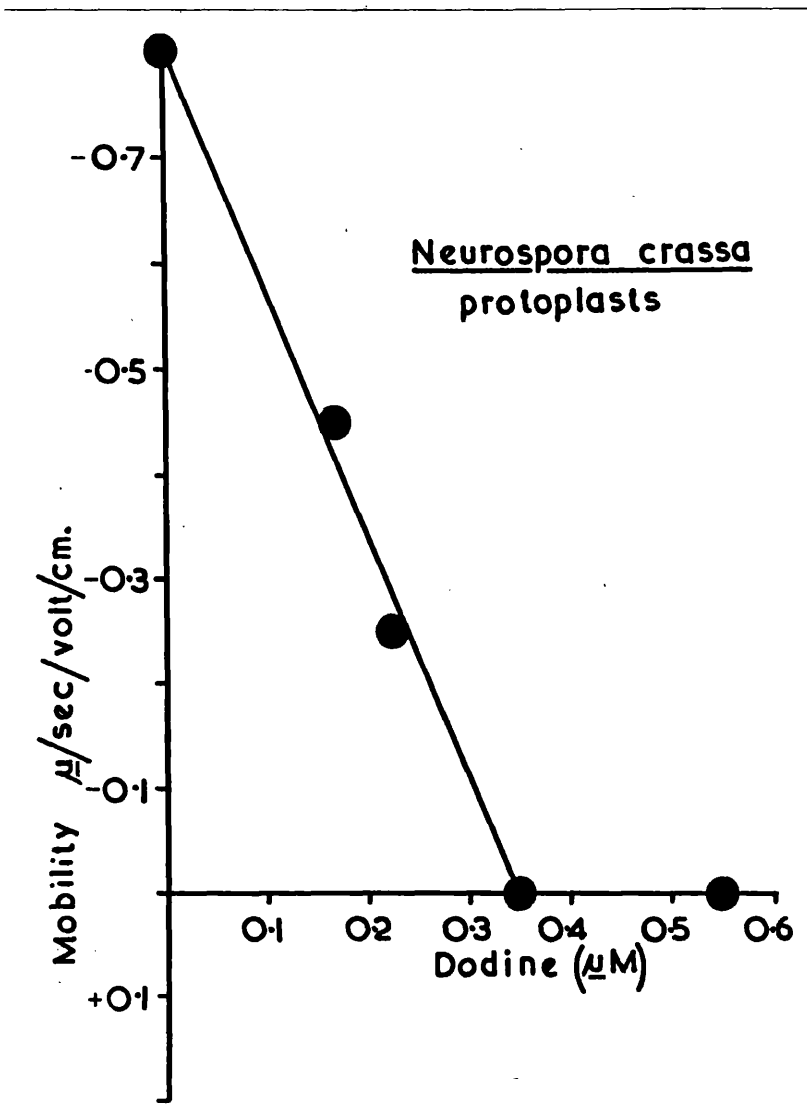


Figure 34. The effect of dodine on the electrophoretic mobility of hyphal protoplasts of Neurospora crassa (0.2 million/ml) in 0.58M-sucrose buffered with acetate to pH 5.6 (I:0.05).

0.4μM dodine the mobility of the conidia was unaffected; in fact even at 4μM no significant reduction in conidial mobility occurred. The fungicidal ED 50 for spores at 0.2 million/ml was 0.7μM dodine. It is of interest that in the absence of dodine the mobility of protoplasts in the sucrose-acetate medium

was -0.80 μ/sec/volt/cm which corresponds to a mobility of -1.45 μ/sec/volt/cm in buffer alone, assuming a simple inverse relationship between mobility and viscosity with other factors remaining constant. Protoplasts are, of course, instantly lysed in the absence of sucrose but this rough calculation indicates that protoplasts carry a greater negative charge than conidia at the same pH i.e. -0.78 μ/sec/volt/cm for N.crassa conidia (Figure 32). Protoplasts of B.fabae showed a similar reduction in mobility in the presence of dodine (Figure 35) and carry a

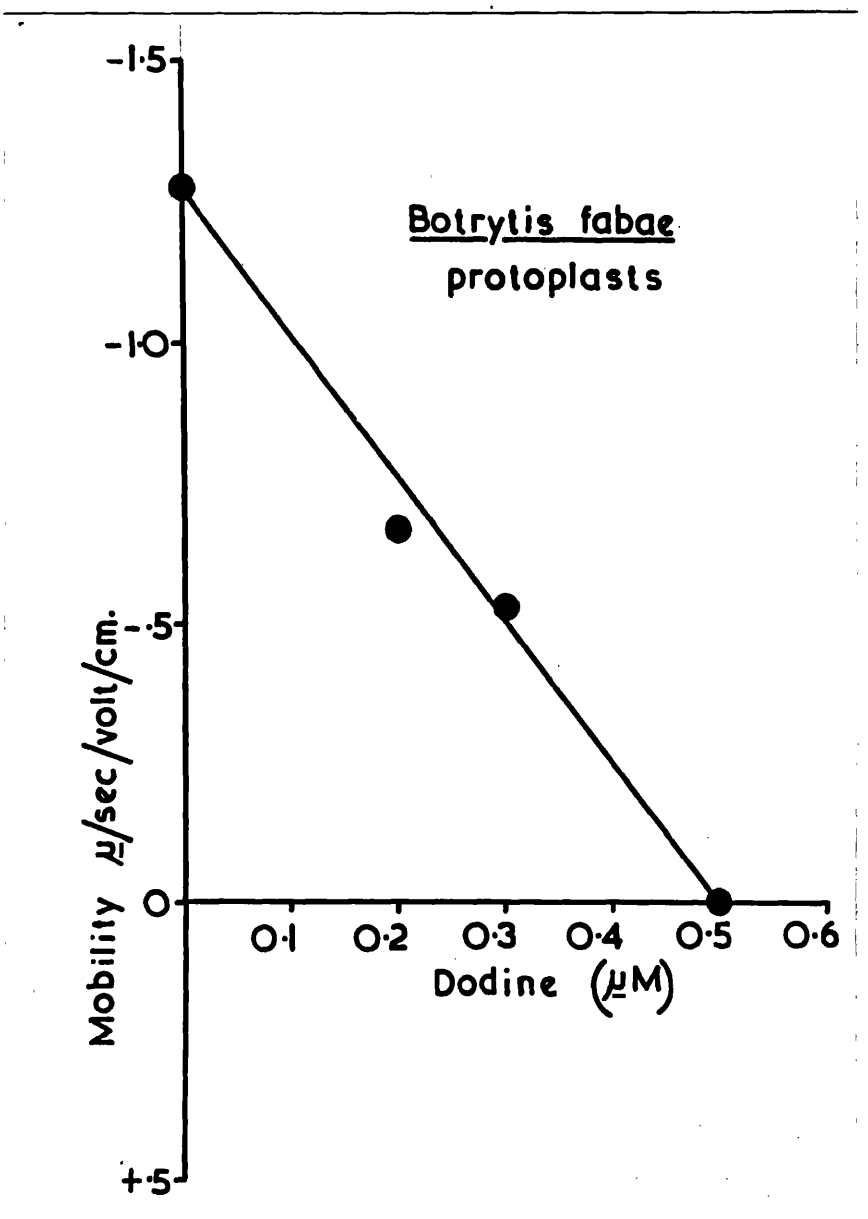


Figure 35. The effect of dodine on the electrophoretic mobility of conidial protoplasts of Botrytis fabae (0.2 million/ml) in 0.58M-sucrose buffered with acetate to pH 5.6 (I:0.05).

greater negative charge than B.fabae conidia at the same pH. Preferential adsorption of sucrose on to the protoplasts, which could decrease the ionogenic area, does not seem likely for there was little difference between mobilities of protoplasts determined in sucrose-acetate in which the sucrose concentration ranged from 0.3 to 1.2M.

The effect of dodine and glyodin on the electrokinetic properties of Botrytis fabae conidia

The effects of increasing concentrations of dodine and of glyodin on the mobility of Botrytis fabae conidia are compared in Figure 36. Glyodin is the more effective in reducing mobility,

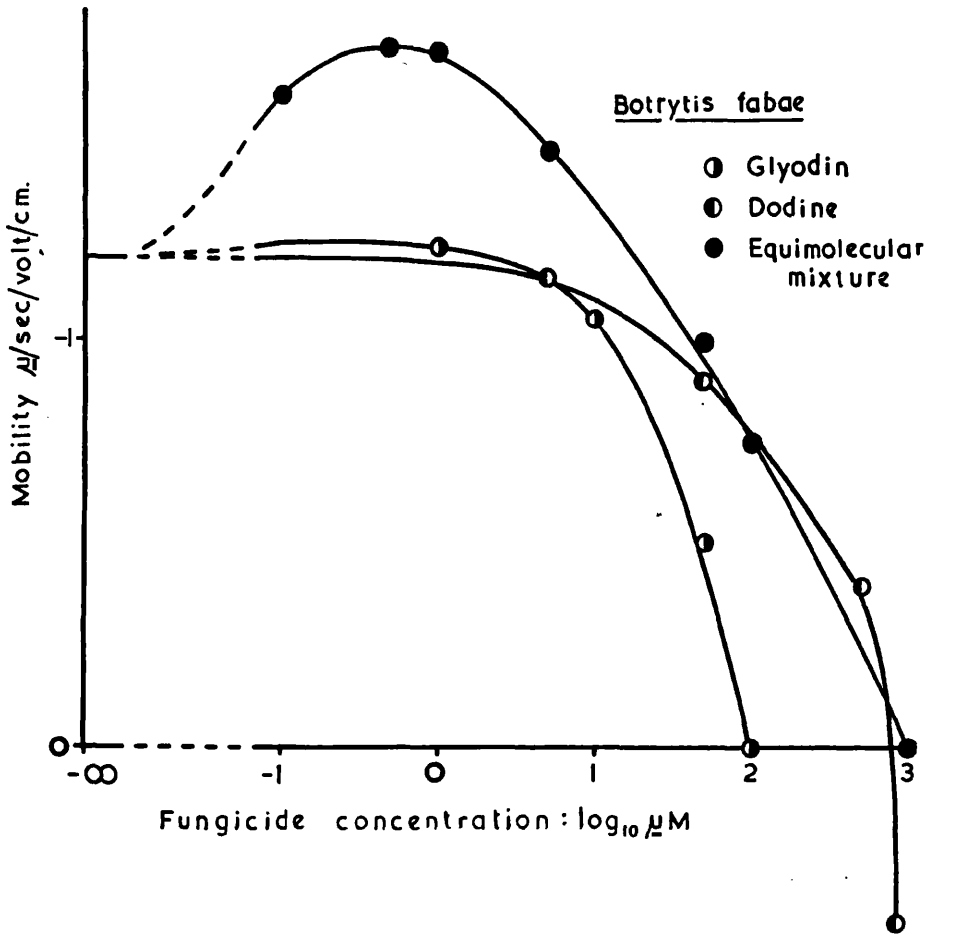


Figure 36. The effect of dodine, glyodin and an equimolecular mixture of the two fungicides on the electrophoretic mobility of Botrytis fabae conidia in acetate buffer (pH 5.6,I:0.05).

and has a lower fungicidal ED 50 concentration (3.2μM) than dodine (7.2μM). An equimolecular mixture of the two fungicides has a fungicidal ED 50 of 1.3μM. Investigation of the effect of such a mixture on spore mobility showed a sharp increase in negative charge at low fungicide concentrations. The maximum negative mobility corresponded to the ED 50 value of the equimolecular mixture.

Discussion

In Part I it was shown that Neurospora crassa conidia carry a negative charge above pH 2.6. Amino, carboxyl and phosphate groups are integral components of the spore surface. At pH 5.6 dodine exists in solution as the positively charged dodine cation which rapidly reacts with surface carboxyl and phosphate groups on the spores. Somers and Pring (1966) have demonstrated that the uptake of dodine by spores increases with increasing ionisation of these groups. Cationic surface-active agents reduce the negative charge on bacteria and, with increasing concentration, completely neutralise the charge, finally reversing mobility to give a stabilised positive charge (Dyar and Ordal, 1946; McQuillen, 1950a). Essentially the same pattern was observed for the interaction of dodine with Neurospora crassa conidia. The charge on the spores is not completely neutralised until they are saturated with the fungicide. After neutralisation, dodine cations can be held by van der Waals forces to surface lipid or to the hydrocarbon chains of fixed dodine molecules so that a positive charge builds up on the spores. The decrease in positive charge at dodine concentrations above 650μM supports a suggestion of McQuillen (1950a) that concentric rings of surface-active agent are built

up round the cell with polar groups facing alternately inward and outward. Spores of Alternaria tenuis showed a similar decrease in mobility in the presence of dodine at pH 5.6. Somers and Pring (1966) showed that cell walls of A.tenuis had greater dodine binding capacity than those of N.crassa. Fungicidal ED 50 values establish that A.tenuis is less sensitive to dodine than N.crassa; this suggests that detoxication takes place at non-essential wall sites. Conidial walls of N.crassa contain approximately five times as much lipid as those of A.tenuis (Part II). Hence, whilst it is possible that dodine molecules are incorporated via their hydrocarbon group into cell wall lipid, this is not the most important site of detoxication. Electrophoretic measurements show that spores of A.tenuis carry a greater negative charge than N.crassa spores, but are more readily reduced to zero mobility by dodine. Ionogenic groups present on the surface of A.tenuis conidia differ from those of N.crassa conidia in having no phosphate component. This may account for the lower surface-binding capacity of the A.tenuis spore.

The major constituents of fungal protoplast membranes are lipids and proteins; carbohydrates and small amounts of other components may also be present (Villanueva, 1966). Fungal protoplasts have been shown in Part I to possess an amino-carboxyl surface indicating an outer protein layer. Somers (1966) considered that cationic fungicides probably bind to negatively charged carboxyl and phosphate groups at or near the cytoplasmic membrane. The present work has shown the anionic charges on N.crassa and B.fabae protoplasts to be neutralised by lower concentrations of dodine than are required to kill conidia. This is consistent

with the suggestion that reaction at the cell wall serves to detoxify much of the accumulated fungicide (Somers and Pring, 1966).

The electrokinetic results provide no evidence that the toxic reaction between dodine and fungal conidia is located on the spore surface. Spores of all the species studied were completely killed before there was a perceptible reduction in mobility. By analogy with surface-active bactericides toxicity might be attributed to disorganisation of the protoplast membrane. Although Brown and Sisler (1960) showed the toxicity of dodine to Saccharomyces pastorianus could be correlated with the loss of vital cell constituents there was almost no lysis of Monilinia fructicola conidia by toxic concentrations of the fungicide. Somers (1963b) found that Neurospora crassa released appreciable concentrations of 260 mμ-absorbing nucleic acid components only when the ED 50 concentration of dodine was exceeded. It seems probable that the reaction of dodine with the protoplast membrane can alter the permeability so as to allow dodine to penetrate into the cytoplasm for the ultimate toxic reaction. Within the protoplast dodine may combine with the mitochondrial and nuclear membranes and destroy their vital functions.

Glyodin is more effective than dodine in reducing the mobility of B.fabae conidia. Spore mobility was completely unaffected by the fungicidal ED 50 concentration: the toxic reaction is thus unlikely to be at the spore surface. In vitro fungicidal tests show a synergistic effect. The sharp increase in negative mobility of B.fabae conidia at the ED 50 concentration of an equimolecular mixture of the two fungicides

shows that a change in surface structure occurs at this concentration. Cells of Staphylococcus aureus show a similar rise in negative mobility followed by a sharp decrease and charge reversal in the presence of increasing concentrations of cetyl-trimethyl ammonium bromide (McQuillen, 1950a). Maximum negative mobility occurred at much higher levels than with the mixture of dodine and glyodin, and corresponds with the saturation of the bacteria by the cationic detergent. The mixture of fungicides may have a more drastic action on membrane permeability than either compound alone. Material released from the cytoplasm by the fungicide mixture may be absorbed back on to the surface causing an increase in negative charge.

C O N C L U S I O N S

CONCLUSIONS

The present studies show the microelectrophoretic technique to provide a convenient means of identifying ionogenic groups on the surface of fungal spores. An uncharged surface probably consisting of cellulose, simple carboxyl surfaces consisting of polysaccharides, amino-carboxyl surfaces derived from protein or amino-sugars and complex phosphate containing surfaces have been identified. Thus the mobility of basidiospores of Stereum purpureum depends entirely on the presence of carboxyl groups; amino and carboxyl groups are present on conidia of Alternaria tenuis and Botrytis fabae while amino, carboxyl and phosphate groups occur on conidia of Neurospora crassa. All the spores examined have characteristic and distinct pH/mobility curves. Spores of similar origin do not necessarily have similar surface components; the surface of uredospores of Puccinia lapsanae is quite different from uredospores of Puccinia pruni-spinosae (=Tranzschelia pruni-spinosae). This shows that closely related species may have spores of widely different surface compositions. Studies of stabilised protoplasts show the plasma membrane has an amino-carboxyl surface. The membrane of fungal protoplasts is probably similar to other cytoplasmic membranes in consisting of a bimolecular leaflet of phospholipid coated with protein (Villanueva, 1966), and the electrophoretic results are consistent with other proposed unit membrane models (Staehelin and Probine, 1970). Prepared cell walls may not have the same surface components as intact conidia due to the loss of labile groups during their isolation.

Surface lipid on fungal spores can be detected by micro-

electrophoresis. Lipid is absent from the surface of most of the spores examined and where present differs in composition from lipid within the spore wall. The sole previous investigation of lipid obtained unequivocally from the surface of fungal spores showed some differences in the fatty acid components of surface and wall fractions (Bertaud et al., 1963). Other lipid components were not investigated. The present work shows the major surface and wall fatty acid components to be the same, though the relative composition of the surface and wall fatty acid fractions differ. Surface and wall hydrocarbon components vary widely in their composition and distribution patterns. Surface hydrocarbons are almost entirely composed of n-alkanes, but wall fractions are more complex. The results demonstrate the presence of a clearly differentiated surface lipid component on some spores, and support the view that some lower plants do not have an odd carbon-number n-alkane preference (Stránský et al., 1966). Phospholipid and minor components of both surface and wall fractions require further investigation. Hydrophobic properties of fungal spores have sometimes been attributed to surface lipids (Horsfall, 1956; Bertaud et al., 1963). All the spores with surface lipids are difficult to wet, but this property is also a feature of some spores from which surface lipids are absent. It is probable that other factors such as the physical conformation of the surface may be important in determining the non-wettable nature of these spores.

Detailed investigations of the surface of Penicillium expansum spores have demonstrated the presence of a superficial polyphosphate component which varies with the composition of

the growth medium and is absent from freshly formed conidia. Differences in the electrokinetic properties of conidia of closely related Penicillium species are explained by the surface polyphosphate layer. In preliminary investigations of Penicillium cyclopium and Penicillium spinulosum spores by the microelectrophoretic technique, Douglas et al. (1959) obtained pH/mobility curves similar to that reported here for unwashed P.expansum conidia. On the basis of comparison with model particles they suggested a wax or lipid surface. Treatment with sodium dodecyl sulphate showed however that lipid was not present on P.expansum conidia. The electrokinetic properties of spores washed free of polyphosphate indicate an amino-carboxyl surface. The composition of the surface rodlet structure on P.expansum conidia has been investigated. In contrast to morphologically similar structures on bacterial spores (Gould et al., 1970) the rodlets are not mainly composed of protein. Rodlets are not completely removed by alkaline extraction although the distinctive amino acid composition of the extract confirms the partial removal of a definite surface layer. The chemical composition of the P.expansum spore does not account for its hydrophobic properties. Non-wettability is not associated with the polyphosphate layer since spores from which this is lacking are still water repellent. The surface is lipid free and, contrary to the suggestion of Hess et al. (1968), does not contain cutin. The non-wettable nature of the surface is probably associated with the physical conformation of the rodlet structure.

The microelectrophoretic technique can be used to follow reaction of cationic fungicides at the surface of fungal spores

and stabilised protoplasts. The surface charge on sucrose-stabilised protoplasts is neutralised at the toxic concentration of the cationic surfactant dodine. The charge on intact spores is not reduced to zero until the toxic concentration is far exceeded. It is evident that the toxic reaction is not situated at the spore surface. Changes at the protoplast membrane are more likely to be the cause of death. Despite the strong affinity of spore walls for dodine (Somers and Pring, 1966) spores are killed long before the wall is saturated. The synergistic effect of dodine and glyodin was confirmed. An increase in charge at the ED 50 value of the mixed fungicides indicated a change in the surface structure of Botrytis fabae conidia at this concentration but further work is required to elucidate the nature of the toxic reaction.

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A P P E N D I X

Appendix 1

MEDIA COMPOSITION

Growth media (Solid)

Alternaria tenuis Carrot agar: 100 g of raw carrot was boiled in water until soft, macerated to a pulp and strained through coarse muslin. 15 g of agar was added and the medium made up to 1 litre with water.

Botrytis fabae Glucose, 40 g; peptone, 10 g; KNO_3, 0.1 g; KH_2PO_4, 6.8 g; $MgSO_4.7H_2O$, 2.5 g; $CaCl_2$, 0.1 g; $FeCl_3.6H_2O$, 0.02 g; agar, 20 g, were made up to 1 litre with water (Hislop, 1967).

Neurospora crassa Sucrose, 20 g; $NaNO_3$, 3 g; KH_2PO_4, 3 g; Na tartrate 1 g; $MgSO_4.7H_2O$, 0.5 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g; Zn as $ZnSO_4.7H_2O$, 2000 µg; Fe as $FeCl_3.6H_2O$, 200 µg; Cu as $CuSO_4.5H_2O$, 80 µg; Mn as $MnSO_4.4H_2O$, 20 µg; Mo as $Na_2MoO_4.2H_2O$, 20 µg; biotin 5 µg; agar, 20 g, were made up to 1 litre with water (Richmond and Somers, 1962).

Verticillium albo-atrum Concentrated prune-extract, 100 ml; lactose, 5 g; Difco yeast extract, 1 g; agar, 30 g, were made up to 1 litre with distilled water. The pH of the medium was adjusted to 5.8 - 6.0. Concentrated prune-extract was prepared by simmering 50 g chopped dried prunes with 1 litre of water until they were soft; the mixture was then strained through muslin and the solution filtered and again made up to 1 litre with water (Talboys, 1960).

Mucor rouxii Tomato-juice agar: 500 ml of commercial tomato-juice plus 25 g of agar were made up to 1 litre with water (Haidle and Storck, 1966).

Liquid medium for the production of yeast-like and filamentous forms of Mucor rouxii

Glucose, 20 g; KH_2PO_4, 3.0 g; $MgSO_4.7H_2O$, 0.5 g; $ZnSO_4.7H_2O$, 1800 µg; $FeSO_4.7H_2O$, 1000 µg; $MnSO_4.4H_2O$, 300 µg; $CuSO_4.5H_2O$, 400 µg; thiamine, 1000 µg;

nicotinic acid,1000 µg, were made up to 1 litre with water. The thiamine, nicotinic acid and glucose solutions were sterilised separately before addition to the inorganic salts; the pH of the mixture was then adjusted to 4.5 with dilute H_2SO_4 (Bartnicki-Garcia and Nickerson, 1962b). The medium was supplemented with 2 g vitamin-free casein hydrolysate per litre (Haidle and Storck, 1966).

Appendix 2

TYPICAL ELECTROPHORETIC RESULTS: CALCULATION AND STATISTICAL ANALYSIS

Mobility of untreated Neurospora crassa conidia at pH 10

Current (i) flowing through the electrophoresis cell = 6.6 mA

Specific conductivity of the buffer = .0044 mhos

Movement right to left		Movement left to right	
Time taken to traverse 180 μ	Velocity μ/sec	Time taken to traverse 180 μ	Velocity μ/sec
10.60 sec	17.0	8.45 sec	21.3
10.30 "	17.5	7.90 "	22.8
9.20 "	19.6	10.05 "	17.9
10.25 "	17.6	9.45 "	19.0
9.80 "	18.4	11.31 "	15.9
9.69 "	18.6	13.25 "	13.6
7.05 "	25.5	9.60 "	18.8
9.70 "	18.6	10.35 "	17.4
16.27 "	11.0	12.19 "	14.8
11.37 "	15.8	10.75 "	16.4

Statistical analysis of particle velocity

mean velocity = 17.89 μ/sec

$$\text{Variance } (s^2) = \frac{n\Sigma x^2 - (\Sigma x)^2}{n\,(n-1)}$$

where n = number of observations

x = individual particle velocities

$$s^2 = \frac{20 \times 6577.45 - (357.5)^2}{20 \times 19}$$

$$= 9.85$$

$$\text{Coefficient of variation} = \frac{100s}{\text{mean velocity}} = \frac{314}{17.89} = 17.56\%$$

$$\text{Standard error of mean} = \sqrt{\frac{s^2}{n}} = 0.702$$

$$\text{Standard error of mean (\%)} = \frac{.702 \times 100}{17.89} = 3.29\%$$

Calculation of electrophoretic mobility

$$\text{Field strength} = \frac{.0066}{.0915 \times .0044} = 16.25 \text{ volts/cm}$$

$$\text{Electrophoretic mobility} = \frac{17.89}{16.25}$$

$$= 1.10 \text{ } \mu\text{/sec/volt/cm}$$

Results were normally calculated using an Olivetti Programma 101 desk computor.

UNITS

This investigation was commenced before the S.I.system came into general use. The c.g.s. system has therefore been used throughout. Mobilities expressed as μ/sec/volt/cm in c.g.s. units are equivalent to 10^{-8} m^2 V^{-1} s^{-1} in the S.I.notation.

DEPARTMENT OF
NATIONAL HEALTH AND WELFARE

CANADA

MINISTÈRE DE LA
SANTÉ NATIONALE ET DU
BIEN-ÊTRE SOCIAL

FOOD AND DRUG DIRECTORATE • DIRECTION DES ALIMENTS ET DROGUES

OUR FILE No.

Nº DE DOSSIER

Tunney's Pasture,
Ottawa 3, Ontario,
November 6, 1970.

TO WHOM IT MAY CONCERN

RE: Mr. D.J. Fisher

Mr. Fisher worked with me from March 1966 until April 1967 at Long Ashton Research Station, University of Bristol. During that time I was Principal Scientific Officer with the status of Lecturer in the University of Bristol.

We studied the effect of the fungicide (dodine acetate) on the electrophoretic properties of Neurospora crassa conidia, and cell components. This work was subsequently published in J. Gen. Microbiol. 48, 147-154, (1967). During the course of these studies to which Mr. Fisher made a substancial contribution theoretically as well as experimentally, Mr. Fisher's relationship to me was essentially that of a post-graduate student.

E. Somers, Ph.D., D.Sc., F.R.I.C.,
Chief,
Food Division.

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The Electrophoretic Properties and Some Surface Components of Penicillium Conidia

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(*Accepted for publication 13 October 1970*)

SUMMARY

Conidia of *Penicillium expansum* are covered with a surface layer of polyphosphate when grown on a high phosphate medium. The composition of this polyphosphate layer, which appears 2 days after conidial initiation, is dependent on the phosphate content of the growth medium; the layer is absent from conidia grown on a low phosphate medium. The rodlet layer which lies beneath the polyphosphate is free of cutin and does not consist of a unique protein. The amino acid composition of the surface protein is, however, different from that of the total wall protein. The rodlet layer appears to be an integral part of the spore wall.

The pH–mobility curves of Penicillium conidia are constant and species-specific when the fungi are grown on defined media.

INTRODUCTION

The chemical composition of hyphal cell walls has been studied in some detail (Bartnicki-Garcia, 1968) but less attention has been paid to the physical and chemical properties of the spore wall. The composition of sporangiospore walls of *Mucor rouxii* (Bartnicki-Garcia & Reyes, 1964) and conidial walls of *Aspergillus oryzae* (Horikoshi & Iida, 1964) have been investigated, while Rizza & Kornfeld (1969) have compared the carbohydrate and amino acid composition of conidial and hyphal walls of *Penicillium chrysogenum.*

The surface ornamentation of fungal spores may be examined by the replica technique (Bigelow & Rowley, 1968) or by freeze-etching (Laseter *et al.* 1968; Hess & Stocks, 1969). The freeze-etching technique has shown that the surface of Penicillium conidia is covered with a distinctive pattern of rodlets (Hess, Sassen & Remsen, 1968).

Ionizable surface groups on fungal spores can be detected by particle electrophoresis in conjunction with chemical and enzymic treatments; the pH–mobility curves of fungal spores may be species specific. Conidia of *Penicillium expansum* are covered with a phosphate layer easily removed by washing to reveal an underlying amino–carboxyl surface (Fisher & Richmond, 1969).

The purpose of the present paper is to describe further the surface components of *Penicillium expansum* conidia and to determine whether closely related species of Penicillium can be differentiated by their pH–mobility curves.

METHODS

Fungal material. Conidia from 7 day cultures of *Penicillium expansum* Link ex Thom, *P. digitatum* Sacc., *P. roquefortii* Thom, *P. thomii* Maire, kindly supplied by Mr R. C. Codner of the University of Bath, and *P. notatum* Westling (CMI 17969), from the Commonwealth Mycological Institute, were grown on malt agar and harvested as previously described (Richmond & Somers, 1963). Cultures of *P. expansum* were also grown on Fries medium (Richmond & Somers, 1962) containing 0·3, 3·0 and 6·0 g. KH_2PO_4/l. The pH of this medium was adjusted to 6·0. Spore walls were obtained by shaking dense spore suspensions with an equal volume of ballotini (no. 12) in a Mickle disintegrator at 4° for 15 min. (Somers & Fisher, 1967). The centrifuged walls were washed ten times with 10% (w/v) sucrose, five times with 0·9% (w/v) NaCl and five times with water following the technique of Dyke (1964). The final washing was free of u.v. absorbing material.

Examination of water-soluble wall components. Soluble surface material was removed from conidia by repeated washing with water or by using the Dyke (1964) technique (described above). Polyphosphates and sugars were examined in the combined water washings after concentration to 5 ml. in a rotary evaporator at 40°.

Polyphosphates were identified by thin-layer chromatography on starch (Canic, Turčic, Petrovic & Petrovic, 1965). The solvent system was trichloroacetic acid+*iso*-propanol+distilled water containing 2·5 mM-EDTA and 2·5 mM-NH_4OH (5 g.+80 ml.+40 ml.). Polyphosphates were detected by spraying the plate with molybdate-perchloric acid (Hanes & Isherwood, 1949). For the determination of metachromatic activity, polyphosphates were precipitated from the concentrated spore washings by a saturated solution of barium acetate. The precipitate was washed, resuspended in 5 ml. water and shaken with 200 mg. Amberlite resin I R-120 (H^+ form). The barium-free solution was examined for metachromatic activity with 0·006% (w/v) aqueous toluidine blue (Nassery, 1969). The values of E_{530}/E_{630} were then calculated. Total phosphorus was determined by the method of Hanson (1950) after digestion with HNO_3 followed by $HClO_4$.

Sugars in the soluble surface material were examined after hydrolysis in 0·5 M-H_2SO_4 for 12 h. at 105° in a sealed tube. Excess sulphate was removed by precipitation with barium hydroxide and the supernatant solution was passed down columns of Amberlite IR-120 (H^+ form) and 400 (acetate form). Total reducing sugars were determined by the arsenomolybdate method (Chan & Cain, 1966). Individual monosaccharides were identified by paper chromatography. The solvent system was *sec*-butanol+acetic acid+water (70+2+28, v/v). Sugars were detected by spraying the paper with *p*-anisidine (Mukherjee & Srivastava, 1952).

Examination of surface protein. Surface protein was extracted from spores by incubating with urea (7 M, pH 2·8) containing 10% (w/v) 2-mercaptoethanol for 1 h. at 37°. The suspension was cooled and centrifuged, and the spores were washed four times with water then incubated with 0·1 M-NaOH for 15 min. at 4°. The alkaline extract was dialysed against running water overnight (Gould, Stubbs & King, 1970).

Amino acids in the extracted non-dialysable wall material were determined on a Technicon TSM.1 amino acid analyser after hydrolysis with 6 M-HCl at 100° in an atmosphere of N_2 for 21 h.

Total protein. Total protein was determined by the method of Lowry, Rosebrough,

Farr & Randall (1951) after treatment of the walls with 2 M-NaOH at 100° for 30 min. (Shah & Knight, 1968).

Detection of cutin acids. Washed spore walls were refluxed with 1 % ethanolic KOH for 3 h. The fatty acids were extracted with ether, methylated with diazomethane and examined by thin-layer chromatography on Kieselguhr HR using chloroform+ethyl acetate (7+3) as solvent or by gas–liquid chromatography using a Hewlett-Packard 5750 gas chromatograph (Baker & Holloway, 1970).

Freeze-etching. Conidia were suspended in 15 % glycerol for 1 h., centrifuged into a pellet, frozen in liquid Freon 12 at −150° and then treated as described by Moor (1966). Replicas were prepared in a Balzers freeze-etching plant BA 360 M and viewed in an AEI EM6B microscope.

Electrophoretic measurements. The electrophoretic mobilities of conidia were measured in a laterally mounted rectangular cell enclosed in a water bath maintained at 25·0 ± 0·2° (Fisher & Richmond, 1969). Measurements were made on conidia which had been washed once with the appropriate buffer before suspension in HCl+NaCl or barbiturate+acetate buffer (*I*: 0·05) of the required pH (Gittens & James, 1963). Movement was timed over 180 μm. in both directions (current reversal). Each mobility was the mean of at least 20 observations; the standard error of the mean was less than 4 %. Mobilities are expressed as 10^{-8} m.^{2} V^{-1} s.$^{-1}$ (equivalent to μm. cm. V^{-1} s.$^{-1}$ in c.g.s. units).

RESULTS

Electrophoretic properties of Penicillium conidia. The pH–mobility curves of conidia from five species of Penicillium grown on malt agar were all different and characteristic (Fig. 1). The pH–mobility curves were affected neither by repeated reculturing of the fungi nor by storage of conidia in water for up to 2 days. There was no evidence to suggest that suspension of conidia in the acid or alkaline buffers caused any surface denaturation; after suspension in pH 7·0 buffer, mobilities were the same as those of normal control conidia. A single washing in buffer before mobilities were determined was necessary to ensure complete removal of water from the conidia, but this washing had no effect on mobility; conidia shaken dry from culture plates had the same mobility as conidia harvested by the normal procedure.

Effect of age on pH–mobility curve of conidia of Penicillium expansum. The pH–mobility curve of 1 day conidia grown on malt agar had a typical amino–carboxyl shape with an iso-potential point at pH 3·5 (Fig. 2). After 2 days the pH–mobility curve (Fig. 2) showed an acid surface with an iso-potential point of 2·0 closely resembling that from 7 day conidia (Fig. 1). As the shape of the pH–mobility curve showed little change after 2 days, all further tests were carried out on 7 day cultures.

Influence of growth medium on pH–mobility curve. The pH–mobility curve of conidia grown on Fries medium (3·0 g./l. KH_2PO_4) was quite different from that of conidia grown on malt agar and indicated an amino–carboxyl surface (Fig. 3). When the phosphate content of the Fries medium was reduced to 0·03 g./l. KH_2PO_4 the pH–mobility curve had a similar shape although mobilities were higher above pH 7·0. When the phosphate content of the medium was increased to 6·0 g./l. KH_2PO_4 an entirely acid surface was formed (Fig. 3).

Effect of washing on pH–mobility curve. The surface of conidia grown on malt agar became progressively less acid as the conidia were washed, until a typical amino–

carboxyl surface was revealed (Table 1). The rapid increase in iso-potential point from 2·0 to 3·4 after only five washings shows that most surface phosphate was easily removable. Further washings produced only a small increase in iso-potential point, but the large increase in positive mobility at pH 2·0 and the similar increase in negative at pH 5·0 suggests that prolonged washing removed non-ionogenic material. When conidia grown on Fries medium with the highest phosphate content were washed the

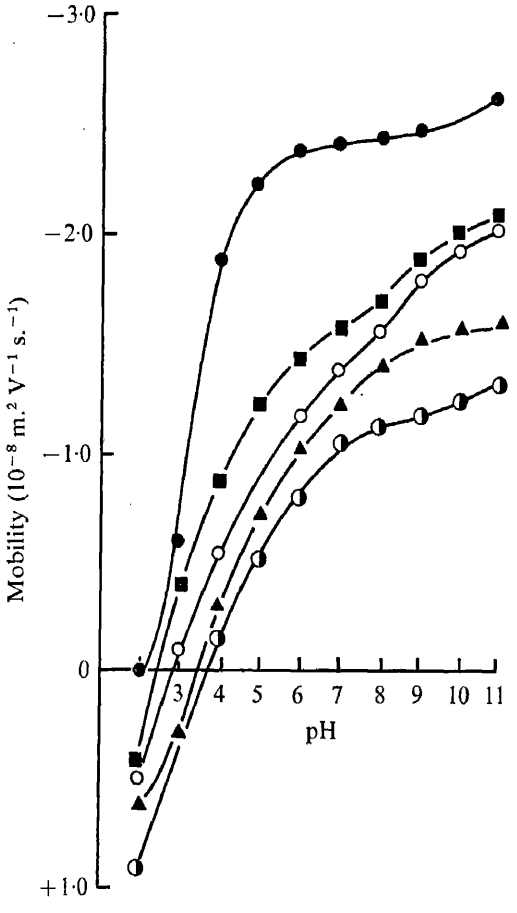

Fig. 1

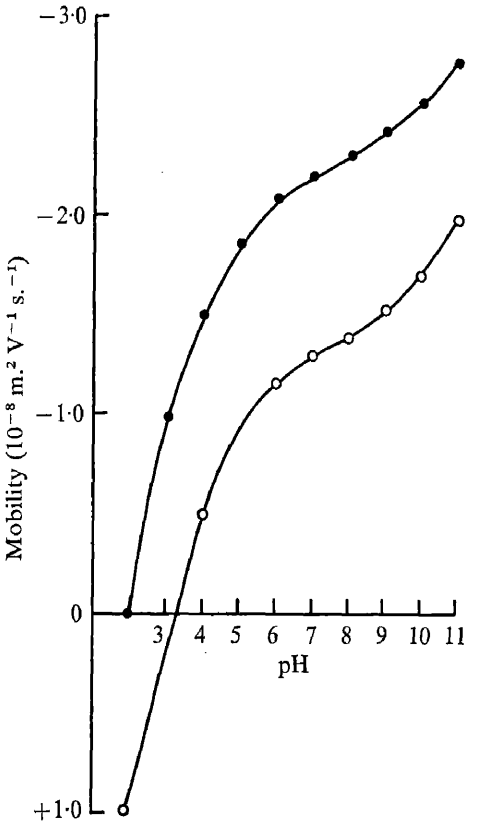

Fig. 2

Fig. 1. pH–mobility curves of *Penicillium expansum*, ●——●; *P. thomii*, ■——■; *P. roquefortii*, ○——○; *P. digitatum*, ▲——▲; and *P. notatum*, ◑——◑, 7 day conidia from malt agar.

Fig. 2. pH–mobility curves of *Penicillium expansum*, 1 day conidia, ○——○; 2 day conidia, ●——●, from malt agar.

Table 1. *Mobilities of washed conidia of Penicillium expansum grown on malt agar*

Washing procedure	Iso-potential point (pH)	Mobilities (10^{-8} m.^{2} V^{-1} s.$^{-1}$) pH 2·0	pH 3·0	pH 4·0	pH 5·0
Control unwashed	2·0	0·00	−0·60	−1·87	−2·23
Water (×5)	3·4	+0·25	+0·08	−0·13	−0·36
Water (×10)	3·5	+0·55	+0·24	−0·21	−0·42
Water (×15)	3·6	+0·63	+0·37	−0·33	−0·71
10 % Sucrose (×10) 0·9 % NaCl (×5) Water (×5)	3·7	+1·08	+0·77	−0·55	−1·10

acid surface was only partially removed even after prolonged washing (Fig. 4). The acid groups appeared to have become an integral part of the surface possibly because the polyphosphate was present in a higher polymeric form.

Composition of spore washings. The amount of phosphorus compounds washed from *Penicillium expansum* conidia varied widely with the nature and phosphorus content of the medium (Table 2). The small amount of phosphorus removed from conidia

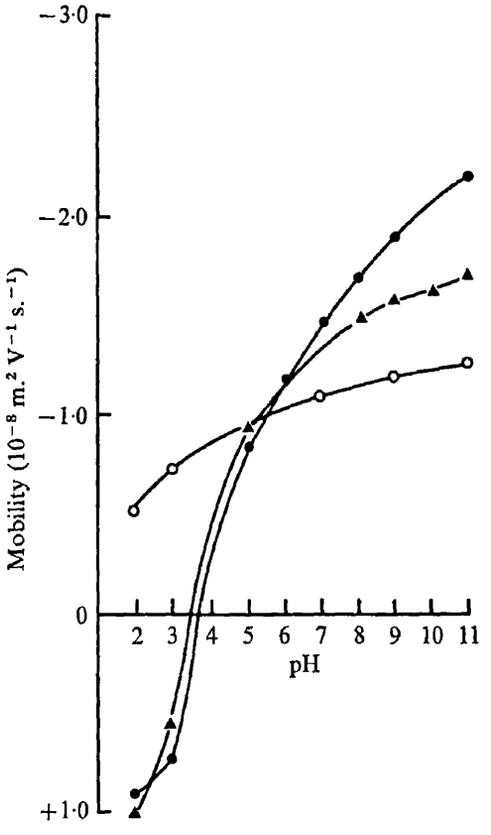

Fig. 3

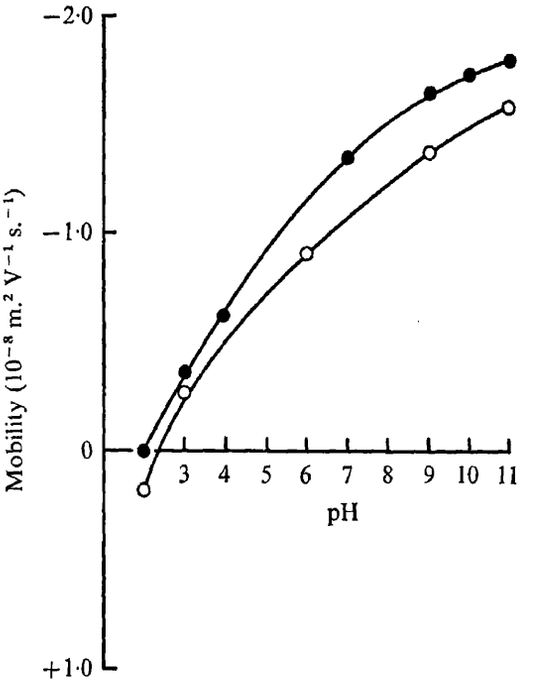

Fig. 4

Fig. 3. pH–mobility curves of *Pencillium expansum* grown on Fries medium, containing 0·03 g./l. KH_2PO_4, ●——●; 0·30 g./l. KH_2PO_4, ▲——▲; 6·0 g./l. KH_2PO_4, ○——○.

Fig. 4. pH–mobility curves of *Penicillium expansum* grown on Fries medium containing 6·0 g./l. KH_2PO_4, conidia washed ten times with 10 % (w/v) sucrose, five times with 0·9 % (w/v) NaCl and five times with water, ●——●; conidia washed twice by above procedure, ○——○.

grown on Fries medium with the highest phosphate content is in agreement with the electrophoretic results which showed that even prolonged washing had little effect on the acid surface (Fig. 4). The metachromatic activity of the washings established that polyphosphates were present on all conidial surfaces. The highest polyphosphate content occurred on conidia grown on malt agar but small amounts were present on conidia grown on Fries medium. Conidial production was greater on malt agar than on Fries medium, even when the phosphorus contents of the two media were similar. Thin-layer chromatography of the concentrated water washings from conidia grown on malt agar confirmed the presence of polyphosphates and showed them to contain less than ten phosphorus atoms. The extracted material was free of nucleic acid, having

no absorption at 260 nm., and imidophosphate linkages were absent as no infrared absorption peak occurred at 7·15 μm. (Correll, 1966).

As prolonged washing of conidia grown on malt agar seemed to remove non-ionogenic material (Table 1) the water washings were examined for carbohydrates. The total reducing sugars (as glucose) from 20 successive water washes was 7·1 μg./g. dry wt of spores before hydrolysis and 12·6 μg./g. after. Glucose and xylose were present before hydrolysis and, in addition, arabinose after hydrolysis.

Table 2. *The effect of growth medium on the phosphorus content of conidia and conidial washings from Penicillium expansum*

Conidia were grown, harvested and washed as described in the text.

Medium	Phosphorus (g./l.)	Dry wt of conidia* (mg.)	Dry wt of conidia (mg./g.) Phosphorus in conidia	Phosphorus in conidial washings	Phosphorus removed by washing (%)	Meta-chromatic activity of washings†
Malt agar	1·33	810	10·7	0·15	1·4	0·17
Fries	0·07	74	9·8	—‡	—‡	0·05
	0·70	584	9·3	0·07	0·8	0·04
	1·40	561	9·3	0·04	0·4	0·03

* From 50 plates. † E_{530}/E_{630} nm. − blank. ‡ = Not determined.

Nature of the rodlet layer. Hess *et al.* (1968) suggested that the outer rodlet layer on Penicillium conidia might consist of cutin or a similar material, since they found the layer to be removed by treatment with aqueous or ethanolic KOH. None of the hydroxy fatty acids characteristic of cutin (Baker & Holloway, 1970) could be detected in ethanolic KOH wall extracts either by thin-layer or by gas–liquid chromatography. The fatty acids extracted from the cell walls after methylation and analysis by gas–liquid chromatography consisted mainly of palmitic (46·5 % of total peak area), oleic (19·9 %) and stearic (19·5 %) acids, although five other acids were present in small amounts. Although many studies have been made on the lipid composition of fungi (Shaw, 1966) less work has been done on isolated cell walls. Laseter, Weete & Weber (1968) have, however, found that palmitic and oleic acids were the most abundant fatty acids in the surface wax from chlamydospores of *Ustilago maydis*.

The rodlets on freeze-etched Pencillium conidia closely resemble the patterns seen on some bacterial spores (Holt & Leadbetter, 1969). The patterned surface layer on *Bacillus coagulans* spores consists of an alkali-soluble protein which can be removed from the spores by treatments which rupture disulphide bonds (Gould *et al.* 1970). Examination of freeze-etched replicas of *Penicillium expansum* conidia showed that the surface rodlets were not removed by incubation with mercaptoethanol in urea followed by treatment with 0·1 M-NaOH. The rodlets were, however, less distinct after alkali treatment, suggesting that some protein material may have been removed. The electrophorectic mobility of water-washed conidia fell, after alkali treatment, from $-2{\cdot}0$ to $-1{\cdot}16\times10^{-8}$ m.^{2} V^{-1} s.$^{-1}$ at pH 10·0 and from $+1{\cdot}1$ to $+0{\cdot}64\times10^{-8}$ m.^{2} V^{-1} s.$^{-1}$ at pH 2·0. The iso-potential point was the same in control and treated spores. The decreases in mobility confirm that the treatment may have partially removed some protein material. Analysis of the non-dialysable alkali-soluble material showed it to contain 11·2 % protein. The remainder of the extracted material probably consisted

of polymeric polysaccharides (Grisaro, Sharon & Barkai-Golan, 1968). The extracted protein which represents only a small proportion of the total conidial wall protein (Table 3) had a high tyrosine and methionine content, proline was absent, and threonine, leucine, isoleucine, histidine, valine and cyst(e)ine were low as compared with conidial wall protein (Table 4). The distinctive amino acid composition of the extracted protein confirmed the partial removal of a definite surface layer. The total conidial wall protein was similar in composition to the cytoplasmic protein. Mercaptoethanol pretreatment had little effect on the removal of surface protein and the amino acid composition of the alkaline extract was the same whether mercaptoethanol was used or not.

Table 3. *Some components of conidial walls of Penicillium expansum grown on malt agar*

	%
Protein	7·67
Phosphorus	0·23
Nitrogen (by Kjeldahl)	3·32
Alkali-soluble surface protein	0·37
Other alkali-soluble material	2·92

Table 4. *Amino acid components of Penicillium expansum conidia*

	Mole ratio		
Amino acid	Alkaline extract from conidia	Conidial wall	Cytoplasm
Aspartic acid	1·38	1·25	1·24
Glutamic acid	1·48	1·13	1·11
Threonine	0·50	1·07	0·84
Serine	1·00	1·10	1·14
Alanine	1·00	1·00	1·00
Glycine	1·13	1·02	0·85
Leucine	0·49	0·76	0·74
Phenylalanine	0·40	0·31	0·40
Lysine	0·26	0·31	0·20
Isoleucine	0·18	0·39	0·23
Arginine	0·43	0·50	0·48
Tyrosine	1·09	0·31	0·36
Proline	n.d.	0·77	0·51
Histidine	0·30	0·61	0·53
Valine	0·34	0·72	0·48
Half cystine	0·23	1·30	1·78
Methionine	0·86	0·13	0·13

n.d. = Not detected.

DISCUSSION

It is at first sight surprising that closely related species should have such widely different mobility curves; the explanation probably lies in the presence of a polyphosphate layer which is not an integral part of the conidial surface.

The presence of surface phosphate on conidia of *Penicillium expansum* was previously demonstrated by treatment with acid phosphatase and confirmed by the decrease in mobility at pH 7·0 which occurred in the presence of Ca^{2+}. Removal of phosphate by enzyme action or prolonged washing revealed an underlying amino–carboxyl surface

(Fisher & Richmond, 1969). The curves of the other Penicillium species (Fig. 1) are indicative of amino–carboxyl surfaces containing varying amounts of phosphate. The curve of *P. notatum* is remarkably similar to that of *P. expansum* after complete phosphate removal (Fisher & Richmond, 1969).

No imidophosphate linkages or nucleic acids were detected in extracts from *Penicillium expansum* conidia and phospholipids are absent as the surface is lipid-free (Fisher & Richmond, 1969). Although surface phosphate groups have been detected on yeast cells (Eddy & Rudin, 1958) and *Neurospora crassa* conidia (Somers & Fisher, 1967) as well as on *P. expansum* conidia (Fisher & Richmond, 1969), the exact nature of the phosphate was not specified. Rothstein & Meier (1951) have, however, suggested that uranyl ions may react with polyphosphate-like groups on the yeast surface, and Harold (1962) has shown that cytoplasmic polyphosphate can bind to hyphal walls of *N. crassa*.

The Penicillium conidium has a three-layered wall covered with an outer patterning of rodlets (Sassen, Remsen & Hess, 1967). Carbon replica studies suggest that the rodlets are themselves covered with an additional very thin film (Hess *et al.* 1968). This film may constitute the polyphosphate layer present on unwashed conidia. Our results show that the polyphosphate layer is not an integral part of the conidial surface. Eddy & Rudin (1958) also found that phosphate groups were absent from the surface of cells grown in phosphate-deficient media.

No specific function can, at present, be suggested for the surface polyphosphate which may act simply as an inorganic phosphate reserve (Harold, 1966). Alternatively, polyphosphate may be implicated in phosphorylation reactions involved in the transport of glucose into the cell on germination (Rothstein & Meier, 1951).

The presence of free xylose in the carbohydrate layer of conidia is of interest. Xylose has been found in hyphal walls of *Penicillium chrysogenum* (Hamilton & Knight, 1962), *P. digitatum* and *P. italicum* (Grisaro *et al.* 1968). Rizza & Kornfeld (1969) were, however, unable to detect xylose in either hyphal or conidial walls of *P. chrysogenum*. Arabinose which was found in the carbohydrate layer after hydrolysis has also been found in small amounts in hyphal walls of *Aspergillus niger* (Johnston, 1965).

The rodlet layer appears to be an integral part of the wall structure since it is not easily separated from the rest of the wall by mechanical disruption. This layer is free of cutin and is not composed of a unique protein. The surface layer does, however, contain protein of a different amino acid composition from that present in the whole wall. The amino acid composition of the whole conidial wall is similar to that of the hyphal wall of *Penicillium notatum* (Applegarth, 1967) except for the presence of valine. Conidial walls of *P. chrysogenum* are, however, quite distinct, since tyrosine, phenylalanine, methionine and histidine are absent (Rizza & Kornfeld, 1969). Fungal cell walls have frequently been reported to contain a full complement of amino acids (Crook & Johnston, 1962; Shah & Knight, 1968; Aronson & Fuller, 1969).

The high tyrosine and methionine content of the surface protein may be significant. Tyrosine is a precursor of melanin, which can protect fungi from enzymic lysis (Kuo & Alexander, 1967; Bull, 1970), while methionine as *S*-adenosylmethionine, an important methyl donor in plants (Meister, 1965), may detoxify injurious substances. Although the rodlet layer superficially resembles the surface layer of *Bacillus coagulans* spores (Gould *et al.* 1970), the two layers are different in structure and composition.

Water repellency can probably be attributed to the rodlet layer. The polyphosphate

layer does not contribute materially to the water-repellent properties of the spores since conidia grown on low-phosphate media lack polyphosphate and are still water-repellent.

The Penicillium spore surface has previously been shown to be lipid-free (Fisher & Richmond, 1969); the present work has not found any other substances which could be responsible for water-repellency. The physical conformation of the surface may itself be sufficient to prevent wetting.

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Evidence for a Surface Protein Layer on the *Saccharomyces cerevisiae* Ascospore

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Electrophoretic measurements on *Saccharomyces cerevisiae* ascospores indicated the presence of a surface protein layer which can be removed by papain, chymotrypsin or 8 M urea.

Little is known about the composition of the outer layers of the yeast ascospore. Because of the hydrophobic nature of yeast ascospores and their affinity for Sudan Black, several workers (3, 9, 13) suggested that the outermost layer is composed of lipid. This conclusion is not consistent with the presence, on the outside of all yeast ascospores so far examined (5, 8), of an electron-dense layer or with the marked ultraviolet-absorbing properties of this layer (12). This report deals with the electrophoretic properties of ascospores from *Saccharomyces cerevisiae* before and after treatment with various reagents. The data suggest that the yeast ascospore is covered with a layer of protein which overlays a thick spore wall probably composed of polysaccharide.

The strain of *S. cerevisiae* (DCL 740) was grown in the presporulation (nutrient broth plus 5% glucose and 1% yeast extract) and KCl (1.0%)-sodium acetate (0.5%) sporulation media recommended by Fowell (5). Approximately 60 to 65% of the cells sporulated after 5 days of incubation at 25 C in the sporulation medium. Asci and vegetative cells were harvested by centrifugation at 0 C and washed twice with water. A suspension of cells and asci (80 mg, dry weight, per ml of 50 mM sodium acetate buffer; *p*H 5.5) was supplemented with one-third volume diluted snail juice (7) and incubated at 30 C for 24 hr. The cells and asci were harvested, and the ascospores were released from asci by subjecting a cold-water suspension to sonic treatment for 3 min with an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd.) at 20 kc per sec. Release of ascospores from asci was monitored by microscopic examination.

Figure 1 shows the *p*H-mobility curve for untreated ascospores. The shape of the curve is indicative of an amino-carboxyl surface, probably of protein (14). Further evidence against the presence of a lipid surface came from the finding that the mobility of spores was not affected by incorporating sodium dodecyl sulfate (0.1 to 0.001 mM) in 0.01 M phosphate buffer (*p*H 7.0) (4). Digestion of spores with trypsin did not alter the shape of the *p*H-mobility curve, although it increased the mobility values at high and low *p*H values. Digestion with pepsin or chymotrypsin changed the electrophoretic mobility pattern to one characteristic of a negatively charged surface (Fig. 2). A similar effect was produced after treatment of isolated spores with 8 M urea (Fig. 2). Electron micrographs of thin sections through ascospores showed that 8 M urea completely removed the electron-dense layer surrounding the spores. Ascospores which had been treated with papain or chymotrypsin retained small amounts of electron-dense material on the surface.

These data suggest that the outside of the ascospores from *S. cerevisiae* is coated with protein, a conclusion which is in agreement with the electron-dense (5.8) and ultraviolet-absorbing (12) properties of this layer. However, the data do not preclude the possibility that the outer layer is composed of a lipoprotein, the lipid moiety of which lies below the surface of the spore. The hydrophobic character of the yeast ascospore suggests that the surface protein may resemble the structural protein found in mitochondrial membranes (1). Marquardt (10) reported that the outside layer of the yeast ascospore is synthesized by the ascal protoplasm rather than by the spore. The electrophoretic mobility of ascospores after digestion with pepsin or chymotrypsin, or treatment with 8 M urea, suggests that the underlying electron-transparent spore wall is probably composed of polysaccharide which is not covalently linked to the surface protein. Eddy and Rudin (2) showed that

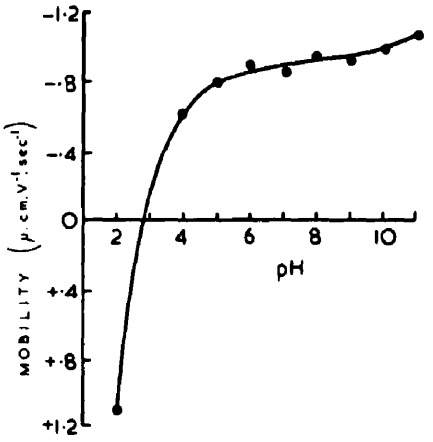

FIG. 1. *Effect of pH value on electrophoretic mobility of ascospores from Saccharomyces cerevisiae DCL 740. Electrophoretic mobilities were measured by a modification of the technique of Somers and Fisher (14). Movement was timed over 180 μm in both directions (current reversal), and each mobility value was obtained from at least 20 observations. The standard error of the mean was less than 4%. Electrophoretic measurements were made by using suspensions containing about 10^6 ascospores/ml. Spores were washed twice in the appropriate buffer before suspension in the HCl-NaCl or barbiturate-acetate buffer of the required pH value (6). All the buffers had an ionic strength of 0.05.*

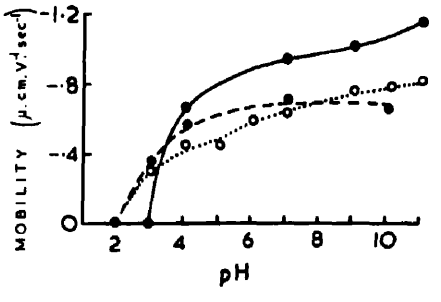

FIG. 2. *Effect of digestion with pepsin* (O——O) *and chymotrypsin* (●——●) *and treatment with 8 M urea* (●···●) *on the electrophoretic mobility of ascospores from Saccharomyces cerevisiae DCL 740. Mobility measurements were made as described in the legend for Fig. 1. Enzyme digestion was done by incubating suspensions of washed ascospores (10^6/ml) containing 100 μg of enzyme/ml for 6 hr at 35 C. The spores were washed three times with water before mobility measurements were made. The pH value of the suspension containing pepsin was 2.5 and 7.5 in the suspension containing chymotrypsin.*

all of the yeasts they examined had walls with a net negative charge which may be attributed to the phosphodiester linkages between mannose residues in the cell-surface mannan (11). However, stationary-phase cells and asci of *S. cerevisiae* DCL 740 have no net charge which suggests that their surface layers differ in composition from the ascospore wall. The known variability of mannose:phosphate ratio in the mannans of different strains and species of yeast could well account for the lack of charge in DCL 740.

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Effect of Dodine Acetate on the Electrophoretic Mobility of *Neurospora crassa* Conidia

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SUMMARY

The electrokinetic behaviour of intact conidia and cell walls of *Neurospora crassa* was studied using a micro-electrophoresis technique. By chemical and enzyme treatments it has been established that amino, carboxyl and phosphate groups are integral components of the spore surface; acid phosphate groups, however, were not found on the surface of washed cell walls. The fungicide dodine acetate reduced the negative charge on conidia to zero and, with increasing concentration, gave a positive charge to the spores: at lower fungicide concentrations the negative charge on the surface of cell walls and stabilized protoplasts was also neutralized. These results are consistent with an ionic reaction between the dodine cation and the carboxyl and phosphate groups of the cell. There was no evidence that the toxic reaction between dodine acetate and *N. crassa* conidia is located on the spore surface—the spores were completely killed before there was a perceptible reduction in electrophoretic mobility.

INTRODUCTION

Although the electrophoretic properties of bacteria and erythrocytes have been extensively investigated, little attention has been given to fungi, except for yeasts. The only studies on fungal spores are those of Douglas, Collins & Parkinson (1959) and Hannan (1961). Their work has shown that spores, in common with other micro-organisms, bear a negative charge over a wide pH range of suspending medium, the nature of the ionogenic groups on the spore surface varying with the fungal species. The electrokinetic behaviour of fungal spores deserves study not only to determine the nature of the ionized, or ionizable, surface groups but also because it is with these groups that the initial reaction with a fungicide—which may be an ionic species—occur.

The surface-active agents toxic to micro-organisms are those which are ionic in character and their reaction at the cell surface can be followed very conveniently by micro-electrophoretic techniques—as has been shown by work on bacteria (James, 1965). Dodine acetate (*n*-dodecylguanidine acetate) is a well-established agricultural fungicide: chemically, it has the structure of a cationic surface-active agent and its accumulation by fungal conidia follows, in general, an ionic bonding pattern (Somers & Pring, 1966). The interaction between dodine acetate and the spore surface has been investigated by the micro-electrophoresis of the intact conidia, protoplasts, and cell walls of *Neurospora crassa*. Particular attention has been given to the nature of the charged groups present on the spore surface with which dodine acetate reacts.

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METHODS

Fungus

Conidia from 7-day cultures of *Neurospora crassa*, macroconidial wild-type Em 5297a, were washed and harvested as previously described (Richmond & Somers, 1962). Cell walls were prepared by shaking dense spore suspensions with an equal volume of ballotini (no. 12) in a Mickle disintegrator, kept at 4°, for 10 min. The centrifuged walls, free of whole cells, were washed 10 times with 10 % (w/v) sucrose, 5 times with 0·9 % (w/v) NaCl, and 5 times with water, following the technique of Dyke (1964). After disruption an appreciable proportion of the cell walls still retained the almost complete shape of the intact conidia and these walls were used for micro-electrophoresis. Total lipid was determined by solvent extraction of dried cell walls (*in vacuo* over phosphorus pentoxide) with hot chloroform+methanol (2+1, v/v) followed by hot ether.

Protoplasts were prepared by incubating young hyphae of *Neurospora crassa* with a *Helix pomatia* digestive-juice extract (L'industrie Biologique Française, Gennevilliers, France) as in the method of Kinsky (1962). The only amendment was that the final protoplast suspension was washed and stabilized in 0·58 M-sucrose maintained at pH 5·6 with sodium acetate buffer of ionic strength 0·05: at 4° the protoplasts were stable for up to 2 days in this medium.

Dodine acetate

A sample, m.p. 134–135°, was prepared by ethanol–ether recrystallization from technical-grade material. Stock solutions made up in ethanol were diluted in the experimental aqueous solutions so that the final ethanol concentration was less than 2 % (v/v). Radioactive dodine acetate, of specific activity 6 μC/mg., labelled in the guanidine carbon with ^{14}C, was generously supplied by American Cyanamid Co. Chemical analysis and radioassay of the dodine cation were carried out as before (Somers & Pring, 1966).

Micro-electrophoresis

The electrophoretic mobilities of conidia, cell walls, and protoplasts—all usually at a concentration of 1 million/ml.—were measured in a rectangular closed cell, air-thermostatted at 25·0 ± 0·2°, whose construction and operation is described by Gittens & James (1960). The cell was mounted in the lateral position of Hartman, Bateman & Lauffer (1952) and the symmetry checked. All subsequent measurements were made at the nearer stationary layer: human erythrocytes in 0·067 M-phosphate buffer (pH 7·35) were used to calibrate the cell (Gittens & James, 1960). The conductivity of the buffered suspensions was measured at 25°, on a Wayne-Kerr B221 bridge. Movement was usually timed over 180 μ in both directions (current reversal) and each mean mobility was obtained from at least 20 observations: the standard error of mean was less than 4 %.

Conidia, cell walls, and protoplasts were washed twice with the appropriate buffer before adding to the electrophoresis cell. All buffer solutions were used at a final ionic strength (I) of 0·05. The following buffer solutions were used for pH/mobility curves (Gittens & James, 1963): below pH 2·6, HCl and NaCl; pH 2·6–9·6, NaCl+sodium acetate+sodium barbiturate+HCl; above pH 9·6, NaCl+sodium acetate+

sodium barbiturate+NaOH. All experiments with dodine acetate were carried out in sodium acetate buffer, pH 5·6: dodine acetate was added to the buffered suspensions and electrophoretic observations made within 5 min.

Treatments to modify the surface groups of Neurospora crassa

Phosphatase. Washed conidia and cell walls were suspended in barbiturate buffer (pH 7·9, *I* 0·02) containing 0·0005 % (w/v) alkaline phosphatase (E.C. 3.1.3.1, British Drug Houses Ltd.) at 37° for 1 hr (Hill, James & Maxted, 1963), then centrifuged and washed twice in the final buffer solution.

1-Fluoro-2,4-dinitrobenzene (FDNB). Conidia were washed 3 times in Sorensen phosphate buffer (pH 7·0, *I* 0·05), then suspended in a 0·1 % (v/v) ethanolic FDNB solution containing 0·9 % (w/v) sodium bicarbonate (Gittens & James, 1963). The suspension was agitated for 5 hr at 25° and the conidia subsequently washed 5 times with ethanol before washing with the final buffer solution. The acid hydrolysis, with constant-boiling HCl, of the FDNB-treated conidia and subsequent paper chromatography of the hydrolysates followed the methods of Ingram & Salton (1957).

Diazomethane. Washed cell walls were dried *in vacuo* over phosphorous pentoxide and methylated with diazomethane as described by Best & Durham (1965): diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide (De Boer & Backer, 1954). Methylated and control cell walls were incorporated into KBr discs and the infra-red spectra recorded on a Perkin Elmer 237 spectrophotometer.

Fungitoxicity of dodine acetate

Fungicidal activity was determined by the method previously described (Richmond & Somers, 1962). The spores were incubated with various concentrations of dodine acetate for 30 min. at 25°, washed twice, then diluted to 50,000/ml. in 0·02 % (w/v) sucrose, and germination counts made after 18 hr incubation in a moist chamber at 25°.

RESULTS

Nature of the surface charges on Neurospora crassa conidia

Many workers have shown that the changes in electrophoretic mobility of microorganisms with pH, measured in buffers of constant ionic strength, can be interpreted in terms of the ionogenic groups present at the cell surface (see James, 1965). In the light of these earlier investigations the form of the mobility–pH curve for *Neurospora crassa* conidia (Fig. 1) suggests the presence of strongly acidic groups and free amino groups (which give rise to the point of inflection at pH 10). Phosphate groups are indicated by a pK as low as 2·6 and confirmed by the displacement of the mobility–pH curve when conidia are treated with alkaline phosphatase (Fig. 1). The remaining negative groups after this treatment (pK 4) are probably carboxyl.

There was no evidence, however, that phosphate groups of pK 2–3 were present on the surface of washed cell walls (Fig. 2) and treatment of the walls with alkaline phosphatase did not alter the pK from 4. The mobility–pH plot in Fig. 2 shows that the amino groups—presumably from surface protein—were unaffected by the washing treatments. The total lipid content of the cell walls was 14 %, on a dry weight basis. Electrophoretic measurements on cell walls in the presence of the anionic sodium

dodecyl sulphate (SDS) showed that some of this lipid was situated on the cell surface. In acetate buffer (pH 5·6, *I* 0·05) the mobility of cell walls increased from −0·69 μ/sec./V./cm. to −1·23, −1·59 and −3·64 μ/sec./V./cm. in the presence of 10^{-5}, 10^{-4} and 10^{-3} SDS, respectively.

The mobility towards the anode of *Neurospora crassa* conidia increased after treatment with FDNB, which is consistent with the removal of $-NH_3^+$ groups (Fig. 1). The hydrolysis of FDNB-treated conidia followed by paper chromatographic analysis to identify the N-terminal groups showed only two spots, corresponding to the DNP-derivatives of ϵ-lysine and histidine.

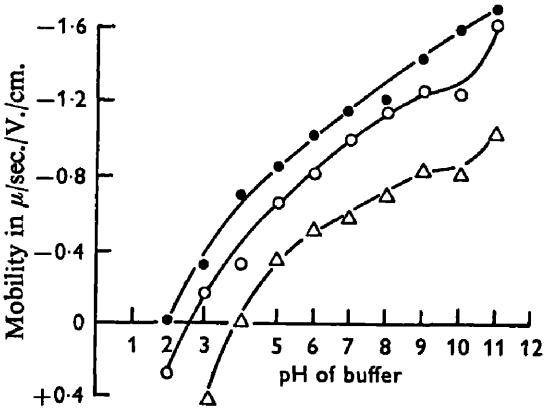

Fig. 1

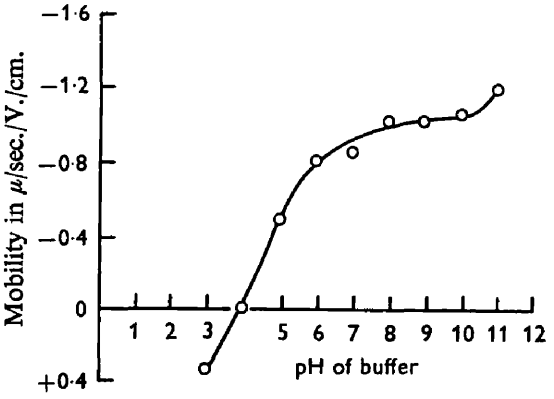

Fig. 2

Fig. 1. Electrophoretic mobility–pH curves for *Neurospora crassa* conidia. ○, Normal conidia; △, conidia treated with phosphatase; ●, conidia treated with FDNB.

Fig. 2. Electrophoretic mobility–pH curve for *Neurospora crassa* cell walls.

Ionized carboxyl groups commonly make a major contribution to the negative electrophoretic mobility of micro-organisms (Douglas, 1959; James, 1965) and their presence at the surface of intact conidia and isolated cell walls has already been presumed from the results following phosphatase treatment. Confirmation has come from the infra-red spectrum of cell walls esterified with diazomethane. Figure 3 shows a pronounced shoulder at 1740 cm.$^{-1}$ for treated walls (curve A) due to the carbonyl stretch of the ester: no such absorption was shown by cell walls subjected to the same preparative treatment, i.e. stirred in methanol–ether for 3 hr, but omitting diazomethane (curve B). Similar results—although with a less pronounced shoulder—have been reported for the bacteria *Aerobacter aerogenes* (Gittens & James, 1963) and *Bacillus subtilis* cell walls (Best & Durham, 1965).

Effect of dodine acetate on Neurospora crassa mobility

Figure 4 shows the changes in electrophoretic mobility of intact conidia and cell walls of *Neurospora crassa* on treatment with various concentrations of dodine acetate at pH 5·6: the dry weight of cell walls, 8 μg./ml., corresponded to 1 million conidia/ml. The negative charge borne by the spores and cell walls was gradually reduced by the dodine cation until at 580 μM for spores and 210 μM for walls it was completely neutralized. At higher dodine acetate concentrations the spores became positively charged. Determinations of the fungicidal activity of dodine acetate showed

that spores, at 1 million/ml., were completely killed after 30 min. immersion in solutions of concentration above 17 μM, i.e. where no effect on the mobility of the spores was apparent; the ED50 was at 5 μM. The uptake of dodine acetate by *N. crassa* spores is known to be very rapid (Somers & Pring, 1966), so that the differences in duration between the fungicidal tests (i.e. 30 min.) and the electrophoretic observations (i.e. up to 5 min.) will not be significant.

When dodine acetate was added to *Neurospora crassa* protoplasts, stabilized in sucrose-acetate medium, there was a rapid reduction in electrophoretic mobility but no evidence of charge reversal (Fig. 5). Protoplasts are obviously much more sensitive to the fungicide than are conidia, for when conidia at the same concentration, i.e. 0·2 million/ml., were incubated with 0·4 μM-dodine acetate the mobility of the conidia was unaffected; in fact even at 4 μM no significant reduction in conidial mobility occurred. The fungicidal ED50 for spores at 0·2 million/ml. was 0·7 μM-dodine acetate.

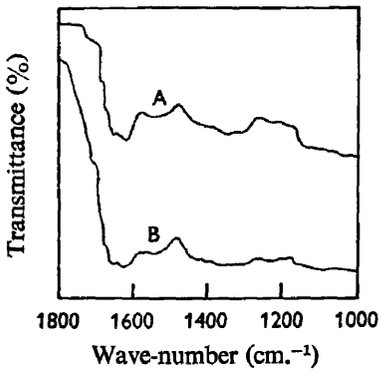

Fig. 3

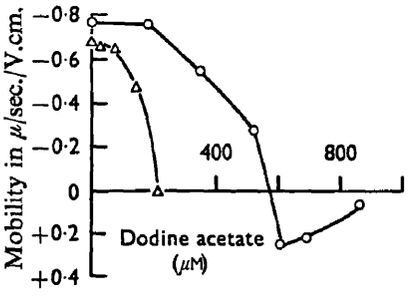

Fig. 4

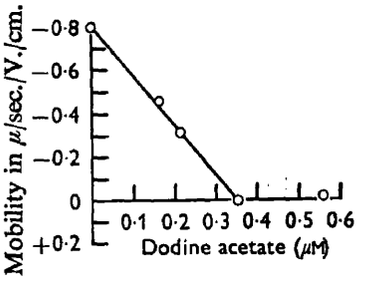

Fig. 5

Fig. 3. The infra-red spectra of *Neurospora crassa* cell walls. A, methylated with diazomethane; B, walls subjected to the same solvent and drying treatment as in A but diazomethane omitted.

Fig. 4. Effect of dodine acetate on the electrophoretic mobility of *Neurospora crassa* conidia and cell walls in acetate buffer (pH 5·6, I 0·05). O, Conidia (1 million/ml.); △, cell walls (8 μg/ml.).

Fig. 5. Effect of dodine acetate on the electrophoretic mobility of *Neurospora crassa* protoplasts (0·2 million/ml.) in 0·58 M-sucrose buffered with acetate to pH 5·6 (I, 0·05).

In the absence of dodine acetate the mobility of protoplasts in the sucrose-acetate medium was $-0{\cdot}80\,\mu$/sec./V./cm., which corresponds to a mobility of $-1{\cdot}6\,\mu$/sec./V./cm. in buffer alone, correcting for the dielectric constant and assuming a simple inverse relationship between mobility and viscosity with other factors such as particle charge and size remaining constant. The protoplasts are, of course, instantly lysed in the absence of sucrose but this rough calculation indicates that protoplasts carry a greater negative charge than conidia at the same pH, i.e. $-0{\cdot}75\,\mu$/sec./V./cm. for conidia (Fig. 1). Preferential adsorption of sucrose on to the protoplasts, which could decrease the ionogenic area, does not seem likely for there was little difference between the mobilities of protoplasts determined in sucrose-acetate in which the sucrose concentration ranged from 0·3 to 1·2 M.

Excess dodine acetate was incubated with protoplasts in the sucrose-acetate medium and, after washing, the protoplasts were completely lysed by transferring to distilled

water. Table 1 shows that 30% of the accumulated dodine cation remained firmly bound to the protoplast membrane. It proved impossible to recover from the protoplast lysate more than a small fraction of the theoretical dodine cation content, presumably due to adsorption of the ion on the walls of the glass apparatus. Dodine cations accumulated by *Neurospora crassa* conidia have been found to be associated with cytoplasmic fractions of the cells (Somers & Pring, 1966) and it is probable that the cell fraction sedimented at 10,000 *g*—which had an appreciable dodine-binding capacity—contained cytoplasmic membrane fragments.

Table 1. *Binding of the dodine cation by Neurospora crassa protoplasts*

Protoplasts, at 7·2 million/ml., were incubated with 10 μM-^{14}C-labelled dodine acetate in 0·58 M-sucrose-acetate buffer (pH 5·6, I 0·05) for 30 min. at 25°, then centrifuged out at 1500 *g* and washed twice in sucrose acetate. The washed protoplasts were suspended in distilled water and the membranes separated by centrifugation at 15,000 *g*. The membranes were washed twice in water before radioassay.

Fraction	Dodine cation content (μmole/10^3 million protoplasts)
Washed intact protoplasts	0·17
Protoplast membranes	0·05

DISCUSSION

In principle the origin of surface charge on *Neurospora crassa* conidia may be due to fixed charges on the cell surface or to adsorbed ions. However, the chemical and enzymic treatments have established that amino, carboxyl, and phosphate groups are integral components of the spore surface and all contribute to the electrokinetic behaviour of the conidia. The surface amino groups have been identified as those of ϵ-lysine and histidine and presumably form part of the protein complexes found in fungal cell wall preparations (Aronson, 1965). Carboxyl groups could originate from the polysaccharide matrix common to the walls of all micro-organisms whilst phosphate groups may be associated with the lipid and nucleic acid components of the *N. crassa* spore wall. It is noteworthy, however, that no acid phosphate groups were found on the surface of purified cell wall preparations, suggesting that the phosphate groups on the surface of intact conidia are removed by the intensive washing used to prepare clean walls. Eddy & Rudin (1958), using a micro-electrophoresis technique, have found phosphate groups on the surface of both intact yeast cells and isolated walls, although it may be relevant that they washed cell walls with distilled water alone rather than with sucrose and NaCl as in the method of Dyke (1964). Certainly Harold & Miller (1961) have shown that the association of polyphosphate with the cell wall of *N. crassa* mycelium is an artifact of the fractionation procedure.

Dyar & Ordal (1946) and McQuillen (1950) have shown that cationic surface-active agents reduce the negative charge on bacteria and, with increasing concentration, completely neutralize the charge, finally reversing mobility to give a stabilized positive charge. Essentially the same pattern was observed for the interaction of dodine acetate with *Neurospora crassa* conidia. At pH 5·6 dodine acetate exists in solution as the positively charged dodine cation and this rapidly reacts with the surface carboxyl and phosphate groups of the spores: previous work has shown that the uptake of dodine cation by spores increases with increasing ionization of these groups (Somers & Pring, 1966). The charge on the spores is probably not completely neutral-

ized until they are saturated with the fungicide. After neutralization dodine cations can be held by van der Waals forces to surface lipid or the hydrocarbon chains of fixed dodine molecules so that a positive charge builds up on the spores. The decrease in positive charge at dodine acetate concentrations above 650 μM (Fig. 4) supports a suggestion of McQuillen (1950) that concentric rings of surface-active agent are built up round the cell with polar groups facing alternately inward and outward.

The saturation level of dodine acetate bound by cell walls of *Neurospora crassa* has been shown to be some 16 % of that retained by intact conidia (Somers & Pring, 1966), hence the charges on cell walls are neutralized by much lower supernatant concentrations than are required for the conidia (Fig. 4). This earlier work also showed the fungicide to be strongly bound to cell walls and it is possible that the dodine molecules are incorporated, via their hydrocarbon group, within the lipid matrix of the cell wall. A wall lipid content of 14 % is fairly high compared with the published values for yeasts and fungi (Phaff, 1963; Aronson, 1965) and the unwettable nature of *N. crassa* conidia suggests that some of the lipid is on the cell surface. Further confirmation of surface lipid has been given by the increased negative mobilities of walls treated with SDS: a result of reaction between surface lipid and the hydrophobic residues of the anionic agent which leaves the polar groups oriented towards the aqueous phase (James, 1965).

The protoplast membrane is apparently similar to other cytoplasmic membranes in consisting of a bimolecular leaflet of phospholipid coated with protein (Villanueva, 1966). The anionic carboxyl and phosphate groups at or near the cytoplasmic membrane of a fungal cell are considered to be binding sites for cationic fungicides (Somers, 1966) and it is probable that the same groups are present on the surface of a protoplast, especially in view of the high negative charge borne by protoplasts. The anionic charges on the protoplast surfaces are neutralized by much lower concentrations of dodine acetate than are required to kill conidia, which is consistent with the suggestion that reaction at the cell wall serves to detoxify some of the accumulated fungicide (Somers & Pring, 1966).

The electrokinetic results presented provide no evidence that the toxic reaction between dodine acetate and *Neurospora crassa* conidia is located on the spore surface —the spores were completely killed before there was a perceptible reduction in mobility. The cytoplasmic membrane may be expected to be extremely vulnerable to the fungicide but toxicity cannot be adequately explained on the basis of physical disorganization of this membrane (Somers & Pring, 1966). Enzyme inhibition at the cytoplasmic membrane or intracellular reaction still appear to be the most probable hypotheses to explain the fungitoxicity of dodine acetate.

We are indebted to Miss C. H. Slowley for providing fungal cultures and to Dr A. M. James for helpful discussions.

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The Electrokinetic Properties of Some Fungal Spores

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SUMMARY

The electrophoretic mobilities of conidia of *Alternaria tenuis*, *Botrytis fabae*, *Penicillium expansum*, *Erysiphe graminis*, *Podosphaera leucotricha* and *Venturia inaequalis*, basidiospores of *Stereum purpureum*, sporangia and encysted zoospores of *Phytophthora infestans* were determined in solution at various pH values. The spores all had characteristic and distinct pH-mobility curves. The zero mobility of *P. infestans* sporangia over the range pH 2 to 11 is consistent with a cellulose surface free from ionizable groups. The mobility of basidiospores of *S. purpureum* depended entirely on the presence of carboxyl groups. Chemical and enzymic treatments showed both amino and carboxyl groups on *A. tenuis* and *B. fabae*; phosphate was present in addition on *P. expansum*. The amino groups of ε-lysine, histidine and leucine contributed to the surface charge of *B. fabae*; amino acids and tyrosine were detected on *A. tenuis*. The surface of *P. expansum* was protein-free and the amino groups present were probably derived from a glucosamine or galactosamine polymer. Washed cell walls and intact conidia of *B. fabae* were electrophoretically similar but cell walls of *P. expansum*, unlike normal conidia, were phosphate-free. Mycelial 'protoplasts' of *A. tenuis* and *Neurospora crassa* and conidial 'protoplasts' of *B. fabae* had pH-mobility curves characteristic of a protein surface.

INTRODUCTION

Douglas, Collins & Parkinson (1959) showed that the electrophoretic behaviour of asexual spores from four fungal species indicated marked differences in the chemical nature of the spore surfaces. Hannan (1961) studied the effect of chemical treatments on the mobility of *Aspergillus niger* spores and Somers & Fisher (1967) investigated in detail the elecktrokinetic properties of conidia, protoplasts and cell walls of *Neurospora crassa*. The latter workers found amino, carboxyl and phosphate groups to be present on the spore surface, but phosphate groups were absent from washed spore walls. The cationic surface-active fungicide dodine (*n*-dodecylguanidine acetate) decreased the negative charge on conidia to zero and with increasing concentration gave a positive charge to the spores. The negative charge on cell walls and 'protoplasts' was neutralized at lower fungicide concentrations.

The present work is part of an investigation into the reaction between fungicides and the components of the fungal spore surface. The electrokinetic properties of spores from the following species have been studied to determine the nature of the ionizable surface groups: conidia of *Alternaria tenuis* Nees, *Botrytis fabae* Sardiña, *Penicillium expansum* Link em. Thom, *Erysiphe graminis* DC. ex Mérat, *Podosphaera leucotricha* (Ellis & Everh.) Salm., *Venturia inaequalis* (Cooke) Wint.; basidiospores of *Stereum*

purpureum (Pers. ex Fr.) Fr.; sporangia and encysted zoospores of *Phytophthora infestans* (Mont.) de Bary. The species include such important plant pathogens as the causal organisms of Potato Blight (*P. infestans*), Apple Scab (*V. inaequalis*), Apple Powdery Mildew (*P. leucotricha*), Oat Mildew (*E. graminis*), Silver Leaf of fruit trees (*S. purpureum*) and Chocolate Spot of broad and field beans (*B. fabae*).

METHODS

Fungal material

Conidia from 7-day cultures of *Alternaria tenuis*, *Botrytis fabae* and *Penicillium expansum* were grown and harvested as previously described (Richmond & Somers, 1963). Sporangia of *Phytophthora infestans* were obtained from cultures grown on potato slices (var. King Edward) at 18°. Zoospores were liberated by incubating sporangia in distilled water for 1 hr at 2°. Conidia of the following species were washed from naturally infected material: *Erysiphe graminis* from oat seedlings (var. Black Supreme), *Podosphaera leucotricha* and *Venturia inaequalis* from apple leaves (var. Cox). Basidiospores of *Stereum purpureum* were obtained from fructifications on pear (var. Hendre Huffcap). Protoplasts were prepared from young hyphae of *Alternaria tenuis* and *Neurospora crassa* wild type Em 5297a, and from conidia of *B. fabae* by incubation with *Helix pomatia* digestive-juice extract (Somers & Fisher, 1967). The final protoplast suspension was washed and stabilized in 0·58 M-sucrose maintained at pH 5·6 with sodium acetate buffer (*I*:0·05). Cell walls were obtained by shaking dense spore suspensions at 4° with ballotini (No. 12) in a Mickle disintegrator (Somers & Fisher, 1967). The centrifuged cell walls, which retained the shape of intact conidia, were washed 10 times with 10% (w/v) sucrose, 5 times with 0·9% (w/v) NaCl and 5 times with water following the technique of Dyke (1964).

Electrophoretic measurements

The electrophoretic mobilities of conidia, cell walls and protoplasts were measured by a modification of the technique previously described (Somers & Fisher, 1967). The laterally mounted rectangular cell was enclosed in a water bath maintained at 25·0 ± 0·2°. The water-immersion objective was focused on the stationary layer through a closely fitting rubber sheet. Washed human erythrocytes in 0·067-M phosphate buffer (pH 7·35) were used to calibrate the apparatus (Gittens & James, 1960). The conductivity of the buffered suspensions was measured at 25°, on a Wayne-Kerr B221 bridge. Movement was timed over 180 μ in both directions (current reversal) and each mean mobility was obtained from at least 20 observations: the standard error of the mean was less than 4%. Electrophoretic measurements were made on conidia, cell walls and protoplasts (1 million/ml.) which had been washed twice with the appropriate buffer before suspension in HCl + NaCl or barbiturate + acetate buffer (*I*:0·05) of the required pH (Gittens & James, 1960). Protoplasts were measured in buffer containing 0·58 M-sucrose.

Treatments to modify surface groups

Alkaline phosphatase: (EC 3.1.3.1). Washed conidia and cell walls were suspended in barbiturate buffer (pH 7·9, *I*:0·02) containing 5 μg. alkaline phosphatase/ml. for 1 hr at 37° (Hill, James & Maxted, 1963).

1-Fluoro-2,4-dinitrobenzene (*FDNB*). Conidia and cell walls were washed 3 times in

phosphate buffer (pH 7·0, *I*:0·05) then suspended in an 0·1 % (v/v) ethanolic FDNB solution containing 0·9 % (w/v) $NaHCO_3$ for 5 hr (Gittens & James, 1963). The sediment was washed 5 times with ethanol before washing with the final buffer solution.

p-Toluenesulphonyl chloride (*PTSC*). Washed conidia in barbiturate buffer (pH 7·0, *I*:0·05) were shaken with 50 mg. PTSC for 24 hr at room temperature (Gittens & James, 1963).

Diazomethane (*DAM*). Washed conidia and cell walls were methylated as previously described (Somers & Fisher, 1967). Conidia were washed once with phosphate buffer (pH 7·0) and twice with HCl (0·05M) before methylation.

Identification of N-terminal amino acid groups

Conidia after treatment with FDNB were hydrolysed in a sealed tube at 105° for 16 hr in the presence of 5·7 N-HCl. After extraction (Biserte, Holleman, Holleman-Dehove & Sautière, 1960) the DNP-amino acids were separated by thin layer chromatography (Pataki, 1967).

Lipid determination

Total lipid was determined by extraction of dried cell walls (*in vacuo* over P_2O_5) with hot chloroform + methanol (2 + 1, v/v) followed by hot ether.

RESULTS

Electrophoretic properties of fungal conidia. Sporangia of *Phytophthora infestans* had zero mobility over the whole pH range (Fig. 1), indicating the absence of surface ionogenic groups. Zoospores of *P. infestans* (Fig. 1) and basidiospores of *Stereum purpureum* (Fig. 2) showed no positive mobility at low pH, suggesting a preponderance of acidic surface groups. The pH-mobility curve of *S. purpureum* is characteristic of a simple carboxyl surface (Hill *et al.* 1963). Cells treated with alkaline phosphatase had a similar mobility to untreated spores, confirming the absence of phosphate groups. Treatment with DAM (Fig. 2) decreased the mobility to zero between pH 2 and 5, suggesting that only carboxyl groups contribute to the surface charge. Above pH 7, however, the mobility did not return to its original value after hydrolysis of the methyl esters. DAM treatment may block other surface groups. Suspension in 0·05M-HCl followed by ether + ethanol and ethanol as required for the chemical treatments did not cause irreversible changes to any of the spore surfaces. After resuspension in pH 7 buffer mobilities were the same as normal control spores.

The pH/mobility curves of *Podosphaera leucotricha*, *Erysiphe graminis*, *Venturia inaequalis*, *Alternaria tenuis* and *Botrytis fabae* (Fig. 2 to 5) are characteristic of mixed aminocarboxyl surfaces (James & List, 1966).

Treatment of *Alternaria tenuis* and *Botrytis fabae* (Fig. 4 and 5) with FDNB decreased the positive mobility at low pH and removed the inflexion at pH 10·0, confirming the presence of amino groups on the untreated surface. Hydrolysis of FDNB-treated *B. fabae* conidia followed by chromatography revealed spots corresponding to the DNP derivatives of ε-lysine, histidine, leucine and an unidentified compound; *A. tenuis* conidia revealed, in addition, a spot corresponding to DNP-tyrosine. The unidentified compound in *A. tenuis* and *B. fabae* had a similar R_F value

to the DNP derivatives of D-glucosamine and D-galactosamine, and might have been derived from a glucosamine or galactosamine polymer in the cell wall.

Cell walls of *Botrytis fabae* gave a pH/mobility curve of the same general shape and with the same isopotential point (3·8) as intact conidia. Conidia of *Alternaria tenuis*,

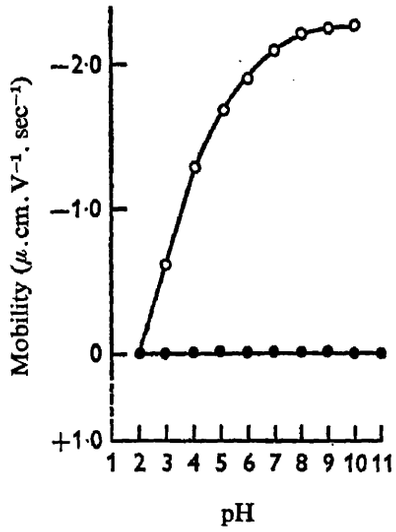

Fig. 1

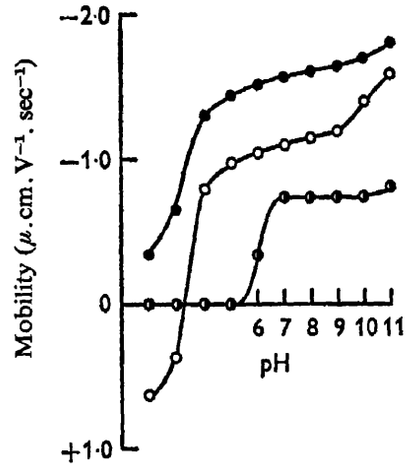

Fig. 2

Fig. 1. pH/mobility curves of *Phytophthora infestans* sporangia, ●—●; encysted zoospores, ○—○.

Fig. 2. pH/mobility curves of conidia of *Venturia inaequalis*, ○—○; basidiospores of *Stereum purpureum*, ●—●; and DAM-treated basidiospores of *S. purpureum*, ◑—◑.

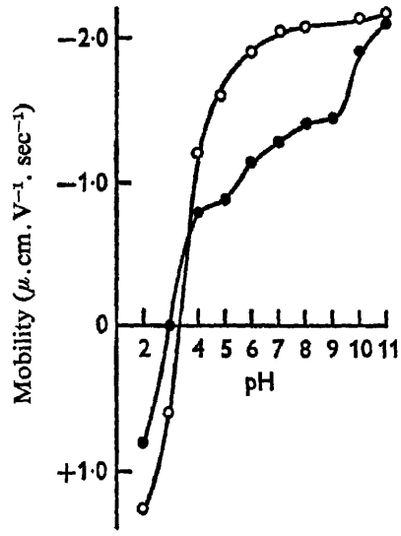

Fig. 3. pH/mobility curves of conidia of *Podosphaera leucotricha*, ●—●; and *Erysiphe graminis*, ○—○.

unlike the other spores examined, are multicellular; this made it difficult to prepare clean cell walls which still retained the shape of intact conidia. Methylation of *A. tenuis* conidia (Fig. 4) and *B. fabae* walls (Fig. 5) with DAM decreased the negative mobility by removing the charge on the carboxyl groups; positive mobilities below

6·0 are due to the remaining amino groups. The decrease in positive mobility of DAM-treated cells below pH 4 may be due to some interaction with amino groups. Mobilities were unaffected by treatment with alkaline phosphatase. Only small increases in the mobility of conidia or cell walls occurred in the presence of sodium dodecyl sulphate (SDS), showing the absence of appreciable amounts of surface lipid (Table 1). The

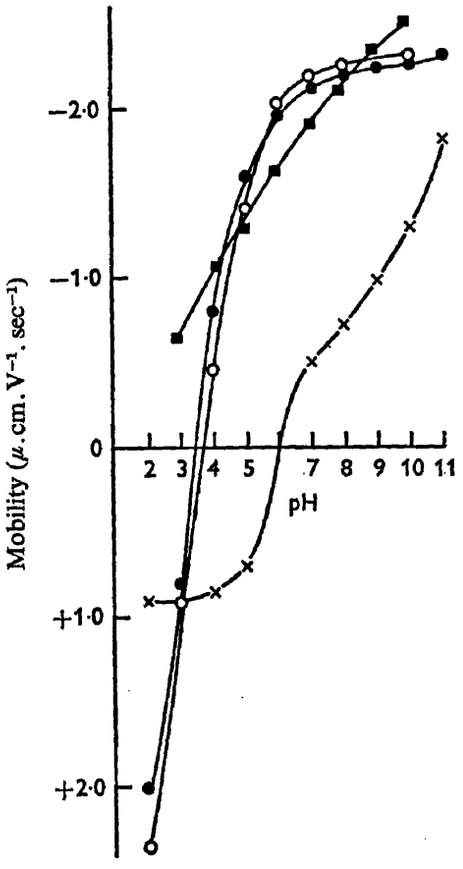

Fig. 4

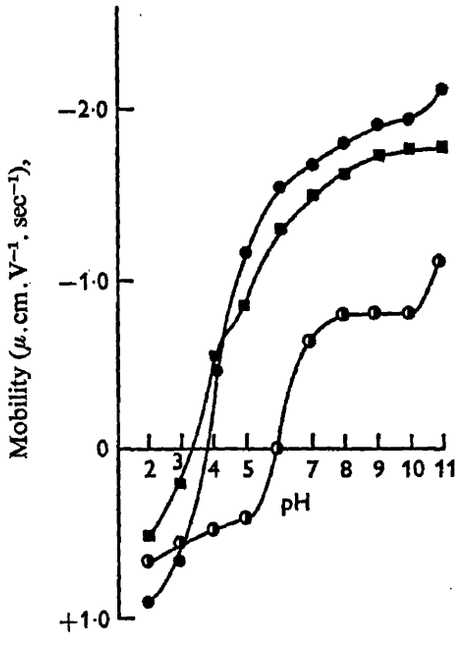

Fig. 5

Fig. 4. pH/mobility curves of conidia of *Alternaria tenuis*. Untreated, ●—●; phosphatase-treated, ○—○; FDNB-treated, ■—■; DAM-treated, ×—×.

Fig. 5. pH/mobility curves of *Botrytis fabae*. Untreated conidia, ●—●; FDNB-treated conidia, ■—■; DAM-treated cell walls, ◑—◑.

Table 1. *Effect of sodium dodecyl sulphate (SDS) on the electrophoretic mobility of conidia and washed cell walls*

Suspension medium: phosphate buffer solution (pH 7·0, *I*: 0·01)

Material	SDS concentration (M)			
	0	10^{-6}	10^{-5}	10^{-4}
	Electrophoretic mobility μ. cm. V^{-1}. sec^{-1}			
A. tenuis (conidia)	−2·62	−3·02	−3·04	−3·26
B. fabae (walls)	−1·41	−1·45	−1·49	−1·53
P. expansum (conidia)	−1·54	−1·80	−1·76	−1·76
P. expansum (walls)	−1·52	−1·42	−1·57	−1·56

total lipid content of cell walls on a dry weight basis was 4·6 % for *A. tenuis* and 6·6 % for *B. fabae*. These figures are much lower than the value of 14 % reported for *Neurospora crassa* (Somers & Fisher, 1967).

The pH/mobility curve for *Penicillium expansum* (Fig. 6) with an isopotential point at pH 2·0 suggests the presence of highly acidic phosphate groups; treatment with alkaline phosphatase (Fig. 6) revealed an underlying amino-carboxyl surface with an

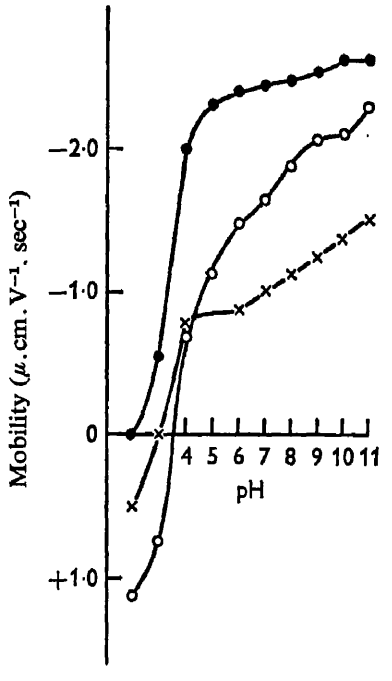

Fig. 6

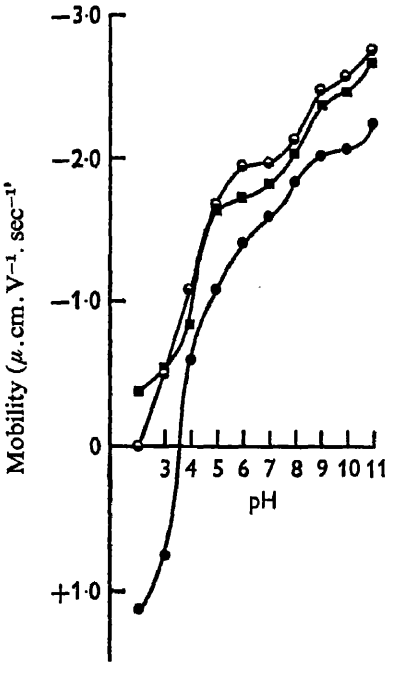

Fig. 7

Fig. 6. pH/mobility curves of conidia of *Penicillium expansum*. Untreated, ●—●; phosphatase-treated, ○—○; DAM-treated, ×—×.

Fig. 7. pH/mobility curves of washed conidia of *Pencillium expansum*, Untreated, ●—●; FDNB-treated, ■—■; PTSC-treated, ◒—◒.

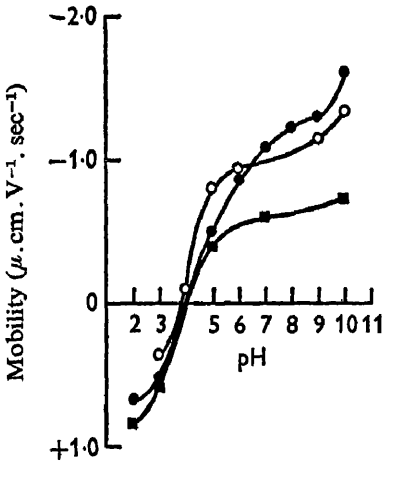

Fig. 8. pH/mobility curves of fungal protoplasts. Hyphal protoplasts of *Neurospora crassa*, ●—●; hyphal protoplasts of *Alternaria tenuis*, ■—■; conidial protoplasts of *Botrytis fabae*, ○—○.

isopotential point of 3·5 similar to untreated *Alternaria tenuis* and *Botrytis fabae* (Fig. 4 and 5). Additional evidence for the presence of phosphate groups on intact *P. expansum* conidia was provided by the decrease in mobility at pH 7 in the presence of 0·01 M-Ca^{2+} from $-2{\cdot}40$ to $-1{\cdot}60$ $\mu.cm.V^{-1}.sec^{-1}$. The washing technique of Dyke (1964), when applied to intact conidia of *P. expansum* (Fig. 7), was as effective as alkaline phosphatase in removing phosphate groups, but extraction with chloroform + methanol (2 + 1, v/v) had no effect on the isopotential point, showing that the phosphate was not present as phospholipid. Indeed, the total lipid content of cell walls was only 1·1 % on a dry weight basis, and mobility measurements in the presence of SDS (Table 1) showed that none of this was present on the surface of either intact conidia or washed cell walls.

Conidia of *Penicillium expansum* treated with DAM (Fig. 6) showed decreased negative mobility due to removal of the charge on the carboxyl groups; after hydrolysis of the methyl ester the mobility was still lower than that of untreated conidia, suggesting that DAM treatment had some blocking action on the amino groups. Interpretation of the pH/mobility curve obtained from FDNB-treated conidia was complicated by the simultaneous removal of some of the labile phosphate component. However, when conidia, washed free of phosphate, were treated with FDNB (Fig. 7) the negative mobility was increased, confirming the presence of amino groups. The inflexion at pH 7 to 8 was not due to secondary amino groups, as it was still present when washed conidia were treated with PTSC (Fig. 7), a reagent which reacts with both primary and secondary amines (Gittens & James, 1963).

Hydrolysis of FDNB-treated conidia followed by chromatography revealed one spot with the same R_F value as the unidentified compound found in *Alternaria tenuis* and *Botrytis fabae*. No spots corresponding to DNP-amino acids were produced, indicating a probable absence of surface protein.

Electrophoretic properties of protoplasts. The pH/mobility curves of protoplasts stabilized in 0·58 M-sucrose from mycelium of *Neurospora crassa* and *Alternaria tenuis* and from conidia of *Botrytis fabae* (Fig. 8) have isopotential points (3·9 to 4·0) typical of a protein surface. (Protoplasts from *B. fabae* represent only a part of the cell contents, being liberated from the germ tube of germinating conidia by constriction of portions of the protoplasm.)

DISCUSSION

All the fungal spores examined have characteristic and distinct electrophoretic behaviours. The sigmoid pH-mobility curves given by protoplasts of *Alternaria tenuis*, *Botrytis fabae* and *Neurospora crassa*, and by conidia of *A. tenuis*, *B. fabae*, *Erysiphe graminis* and *Podosphaera leucotricha* are typical of amino-carboxyl surfaces. Conidia of *Penicillium expansum*, *Stereum purpureum* and encysted zoospores of *Phytophthora infestans* show no positive mobility at low pH, indicating a preponderance of acidic surface groups. Sporangia of *P. infestans* are unusual in having zero mobility over the whole pH range and must be completely lacking in surface ionizable groups. Clean *B. fabae* cell walls are electrophoretically similar to intact conidia but *P. expansum* walls are quite distinct from *P. expansum* conidia: the washing technique removed phosphate groups from *P. expansum* conidia to reveal an underlying amino + carboxyl surface. Chemical treatment of *A. tenuis* and *B. fabae* has shown phosphate and lipid

to be absent from the surface and confirmed the presence of amino and carboxyl groups.

The amino groups on the spore surfaces are probably a part of the protein components of the cell wall (Aronson, 1965; Manocha & Colvin, 1967), although aminopolysaccharides (Harold, 1962) may also be present. Carboxyl groups may derive from proteins, polysaccharides (Applegarth, 1967) or uronic acids (Mitchell & Scurfield, 1967). Somers & Fisher (1967) found ε-lysine and histidine on the surface of *Neurospora crassa* conidia and we have found, in addition, leucine on *Botrytis fabae*, and leucine and tyrosine on *Alternaria tenuis*. The high positive mobility of *A. tenuis* at low pH together with the negative mobility after treatment with FDNB suggests that *A. tenuis* has a higher proportion of surface amino groups than *B. fabae*.

The surface of *Penicillium expansum* resembles that of *Neurospora crassa* (Somers & Fisher, 1967) in having easily removable phosphate groups but differs in having no surface lipid. This is reflected in the much lower total lipid content of cell walls of *P. expansum* (1·1 %) compared with 14 % reported for *N. crassa* by Somers & Fisher (1967). Although no DNP-amino acids were detected in acid hydrolysates from FDNB-treated conidia an unidentified compound was found which could have been the DNP-derivative of glucosamine or galactosamine. This suggests that the surface is protein free but may contain polyglucosamines or polygalactosamines. Harold (1962) has shown that inorganic polyphosphate can bind to protein and polygalactosamine receptor sites on the outer surface of *N. crassa* hyphae: the phosphate groups on *P. expansum* conidia may be bound in a similar way. The observation of Hess, Sassen & Remsen (1968) that a very thin layer is present on the outside of conidia of some Penicillium species when examined by carbon replica techniques may also be relevant here.

As the only ionized groups on *Stereum purpureum* are carboxyl the surface must be protein-free and probably consists entirely of carbohydrate. The absence of ionogenic groups from sporangia of *Phytophthora infestans* is consistent with the work of Chapman & Vujičić (1965), who showed that young sporangia of *Phytophthora erythroseptica* have a structureless, electron-transparent outer layer consisting probably of cellulose.

The protein-like pH/mobility curves of the fungal protoplasts are in agreement with the lipoprotein constitution of fungal cytoplasmic membranes (Villanueva, 1966). The curves are similar to those reported for rat liver nuclei by Vassar, Seaman, Dunn & Kanke (1967) and bacterial protoplasts by James, Hill & Maxted (1965) in conformity with the unit membrane theory (Robertson, 1959).

The water-repellent properties of Penicillium conidia have been attributed to the presence of ether-soluble cyclic peptides on the spore surface (Bertaud, Morice, Russell & Taylor, 1963) or to the characteristic 'rodlets' which may consist of cutin or sporopollenin detected on the surface of freeze-etched conidia by Hess *et al.* (1968). The small amount of material soluble in chloroform + methanol in cell walls and the absence of surface lipid suggest that the water-repellent properties are not due to lipids or cyclic peptides. Further studies in conjunction with chemical treatments are required to elucidate the problem. Powdery mildew conidia are also water-repellent and differ from spores of other fungi in their resistance to desiccation (Yarwood, 1936) furthermore spore germination is usually impaired by wetting (Zaracovitis, 1964). McKeen, Mitchell & Smith (1967), in a study of the *Erysiphe cichoracearum*

conidium, considered that the impervious outer layer of the spore played an important role in the action of water and fungicides on mildew conidia. The pH-mobility curves of *Podosphaera leucotricha* and *Erysiphe graminis* do not account for the characteristic physical properties of powdery mildew conidia: unionized compounds may be responsible for the non-wettable nature of these spores.

Fungal spores frequently have a specialized and complex morphology which differs considerably from the vegetative cells. Electron microscopic studies of germinating spores have shown that the germ tube wall may be continuous with either the inner (Remsen, Hess & Sassen, 1967), or the outer (Manocha & Shaw, 1967) layer of the spore wall, or an entirely new wall may be formed (Hawker, 1966). Several detailed studies have been made of the chemical composition of hyphal cell walls (Aronson, 1965; Rogers & Perkins, 1968), but less attention has been paid to the spore wall, although Horikoshi & Iida (1964) have compared the composition of hyphal and conidial walls of *Aspergillus oryzae* and detailed studies have been made of the chemical structure of the cell wall of *Mucor rouxii* at different stages of development (Bartnicki-Garcia & Nickerson, 1962; Bartnicki-Garcia & Reyes, 1964; Bartnicki-Garcia & Reyes, 1968). Spore walls and hyphal walls of *A. oryzae* were qualitatively identical but the composition of spore walls of *M. rouxii* was markedly different from vegetative cell walls. Differences in cell wall composition may explain why fungal spores differ in their susceptibility to fungicides and why some fungicides are more effective against spores than mycelium, while, with others, the reverse is true (Kreutzer, 1963). The physical properties of the spore surface will influence dispersion by air, water or animals (Gregory, 1966); the chemical composition may modify toxicant action. Studies of the fungal spore surface may make some contribution to an understanding both of fungal ecology and of the mechanisms of the selective toxicity of compounds to spores of different fungal species as well as to spores and mycelium of the same species.

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